PHYSICOCHEMICAL STUDIES ON STARCHES

THE MOLECULAR PROPERTIES OF THE COMPONENTS OF STARCHES OF VARIOUS PLANT ORIGINS

by

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ABSTRACT OF THESIS

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Title of Thesis The Molecular Properties of the Components of Starches of various Plant Origins.

Current concepts in the chemistry of the starch-type polysaccharides with particular reference to their fine structure, have been critically discussed. The enzymic and physical techniques used in this work are detailed and an enzymic assay for the determination of the purity of amylose has been demonstrated.

A critical examination of the factors influencing the purity of the isolated starches, and the methods of fractionating starch has been undertaken. The necessity for an inert atmosphere in starch fractionating procedures has been illustrated, and the fractionation of potato starch by centrifugation in alkali has been completed and shown to be inefficient. The success of the method of fractionating amylose from dimethyl sulphoxide has been demonstrated.

A number of starches from a wide variety of botanical sources have been isolated and characterised. Pretreatment of the granules with liquid ammonia was found to be a general method of ensuring satisfactory fractionation. A comparison of barley and malted barley of the same variety, showed that during the malting process, the amylose and amylopectin components of the barley starch are preferentially attacked by the amylase enzymes. Floridean starch has been isolated, purified by differential ultracentrifugation and shown to have an amylopectin-type structure.

Finally a critical comparison of the components from smooth and wrinkled pea starch has been completed. The changes occurring in the structure of the components of pea and potato starch during growth, have been related to changes in granular size and considered in the light of several hypotheses of starch biosynthesis.

Use other side if necessary.

TO

MY PARENTS

and

HAZEL.

CONTENTS

Preface.

I wish to thank Dr. C.T. Greenwood for the invaluable advice and guidance given to me during the period of this research. Some of the work described in Sections 3, 4 and 5 has been published in conjunction with Dr. Greenwood and reprints of these papers are inserted at the end of this thesis.

I wish to thank Professor E.L. Hirst for the excellent laboratory facilities provided, and the Department of Scientific and Industrial Research for a Maintenance Grant.

I also wish to thank Dr. W. Banks for his collaboration in certain aspects of this work.

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General Introduction

Although there has been considerable progress in the field of starch chemistry during the past twenty years, as yet few starches have been completely characterised. There is in fact a considerable dearth of knowledge regarding changes occurring in the structure of the starch components during growth. Such precise knowledge would be of considerable aid in elucidating the many problems, which prevent the biosynthesis of the starch granule from being fully understood.

The first section of this thesis surveys the general concepts accepted for the structure of the amylose and amylopectin components of starch, and introduces and explains several of the theories of starch biosynthesis. Section 2 deals with the general experimental techniques used in this work and is followed by studies on the fractionation and subfractionation of starch and its components. Section 4 is devoted entirely to the characterisation of starches from several botanical sources. In section 5, a comparison is made of smooth and wrinkled pea starch and the changes occurring in the structure of the components of pea and potato starch during growth are critically studied.

SECTION I

THE COMPOSITION AND BIOSYNTHESIS OF THE STARCH GRANULE.

The Properties of the Components of Starch.

According to the prevailing theories, starch contains two carbohydrate substances: both polymers of glucose, but differing markedly in properties and structure. Schoch (1942) was the first worker in the field of starch chemistry to successfully fractionate starch into its components. These fractions were originally named A- and B-fractions, but these terms have now been replaced by amylose and amylopectin (I.U.P.A.C., J. Polymer Sci., 8, 257, 1952). In most starches, amylose is the minor component. This component appears to consist of chain-like molecules of α-1:4-D-glucopyranoside units. It is unstable in a colloidal sense, and is responsible for the gelation and so called "retrogradation" of starch. The major component (amylopectin) has a highly ramified molecular structure, consisting as in amylose, of chain-like molecules of α-1:4-D-glucopyranoside units with α-1:6 cross linkages.

Opinions regarding the molecular size of the constituent carbohydrates of starch have run the entire gamut of speculation, from the consideration of starch as composed of simple, associated di and tri-hexosan molecules (Pringsheim, 1928) to the concept of the starch granule itself as a single chemical entity. However there is no doubt from the work of Schoch and his collaborators (1942), that starch consists essentially of two fractions, amylose, a straight-chained fraction and amylopectin, a branched-chain fraction. In Table 1.1 are tabulated some of the principle properties of these two components.

Table 1.1

	Amylose	Amylopectin
Molecular configuration	Essentially linear	Branched molecule
Molecular weight	ca. 10^6	ca. 10^8
X-ray diffraction	Crystalline	Amorphous or weakly crystalline patterns
β -amylolysis	ca. 70-80% conversion into maltose	ca. 55% conversion into maltose
Concurrent action of β -amylase + Z-enzyme	Complete hydrolysis	High molecular weight dextrins
Complex formation	Readily forms complexes with I_2 and polar substances	Very limited complex formation with I_2 and polar substances
Stability in aqueous solution	Unstable, tends to retrograde in conc. solution	Stable at any concentration

Amylose

This is the minor component of most starches. It is an essentially linear molecule and is a polymer of D-glucopyranoside molecules joined together by 4 → 1 - α - D glycosidic linkages. In aqueous solution this component tends to aggregate and finally to precipitate (retrograde) from solution. Caesar and Cushing (1941) forwarded the hypothesis that amylose assumes a helical conformation in its stable form, whereas in retrogradation the glucose-chains align themselves to form crystallites, which eventually grow

into visible aggregates. This aggregation observed in amylose is effected by the pH. and concentration of the amylose solution. It is however thought that the size of the amylose molecule has the greatest influence on the rate of retrogradation. Lansky et al (1949) have shown that the retrogradation time of a series of amylose subfractions was inversely proportional to the chain-length until a certain critical value was reached, below which the molecules were too small to crystallise. This retrogradation phenomenon prevents amylose from being maintained in neutral or acid solution. The normal solvent for amylose is alkali. The use of alkali unfortunately introduces the possibility of degradation of the amylose. This alkali degrading effect has been known for some time. Schoch, Wilson and Hudson (1942) concluded from the fact that methyl β-maltoside is stable when heated in 0.1 M caustic soda for considerable periods of time, that alkaline degradation of starch occurs only through the reducing end group. Physical measurements, however, showed that random degradation of $4 \rightarrow 1$-α-D bonds must also occur. For example, if amylose is dissolved in 0.1 M alkali for any length of time at room temperature, the specific viscosity of the solution decreases. Whistler and Johnston (1948) showed that this degradation was reduced to a minimum if the dissolution in alkali was achieved at 0°C. in a nitrogen atmosphere. Gundrum and Rist (1950) have suggested, however, that at 0°C. a nitrogen atmosphere is unnecessary as less than 1% decrease in limiting viscosity number was found for some amyloses after 24 to 48 hours at this temperature. Bottle et al (1953) carried out an extensive study of amylose degradation in

neutral and alkaline solution. They found that potato amylose was stable in aqueous solution, and in 0.2 M alkali at 100°C., if oxygen was excluded. In the presence of oxygen a slow decrease in the limiting viscosity number occurred in aqueous solution and a very rapid decrease in alkali. Baum and Gilbert (1954) have suggested that amylose contains oxygen-sensitive bonds, which in the presence of oxygen, are hydrolysed in hot aqueous solution. Amylose is therefore unstable in the presence of oxygen and for this reason, all fractionations and recrystallisations are best conducted in an inert atmosphere.

Molecular shape of amylose in solution - Relatively few measurements have been carried out on the molecular shape of amylose in solution. Conflicting evidence suggesting that amylose is (a) a rigid rod and (b) a spring- like helix, has been found by several workers. Foster and Hixon (1943) have found the value of α in the modified Staudinger equation (see p. 44) to be equal to 1.5 both for amylose acetates in chloroform solution and for the unsubstituted amyloses in ethylenediamine. This high value of α was interpreted as suggesting that the amylose molecule behaves as a rigid rod in solution. However those results are not in agreement with those of Potter and Hassid (1948) who found $\alpha = 1$, or Goodison and Higginbotham (1950), who have found that the value of α varied between 0.44 and 0.87 for acetates in nitro-ethane. Peterlin (1950) has shown that the viscometric data for potato amylose acetate in chloroform solution can be readily interpreted in terms of a random-coil model for the molecule in which there is hindered rotation at the oxygen atom of the glucosidic linkage.

Ultracentrifugal and viscometric studies of several amylose acetates in methyl acetate solution by Dombrow and Beckmann (1947) were interpreted as supporting the idea of a helical configuration in solution. Banks (1960) determined the shape of amylose in solution by consideration of the viscosity-molecular weight relationship and comparison of the experimental value of α in the modified Staudinger equation ($\eta = KM^{\alpha}$), with the theoretical values derived from the consideration of simple models. The theoretical value of α for a rigid rod type of molecule is ca. 1.7, whereas that for a random coil is between 0.5 and 0.8 depending on the degree of swelling in a particular solvent. Banks experimental value for α were 0.56 and 0.78 in neutral and alkali solution respectively which falls into the range demanded by theory for a randomly-coiled molecule.

Molecular weight distribution. - The subfractionation of potato, maize and tapioca amyloses was studied by Lansky et al. (1949). These workers found that the distribution curves indicated that the samples consisted of a homologous series of linear chains. Meyer, Bernfeld, Boissannas, Gurtler and Noelting (1949) suggested that the molecular weight distribution of potato and maize amyloses are discontinuous i.e. there are two distinct ranges of molecular weight present in amylose samples. However, Bryce (1958) in distribution studies on potato amylose, obtained distribution curves from sedimentation studies which indicated a continuous molecular weight distribution.

The fine-structure of amylose. - Meyer and his co-workers in 1940 first established the essentially linear nature of the amylose molecule. They found that the degree of polymerisation of maize amylose obtained from

methylation studies, agreed well with that derived from osmotic pressure measurements. Hassid and McCready (1943), completely confirmed Meyer's results, but Hess and his co-workers in 1940 suggested that potato amylose was slightly branched. All these results are obviously suspect because of the probability of amylopectin contaminant and the possible degradation which might occur during methylation.

The linear nature of amylose has also been studied using the technique of periodate oxidation. Potter and Hassid (1951) suggested from a comparison of chain-length (from periodate oxidation) with molecular weight, that some amyloses may be branched. As the degree of polymerisation for amylose is probably measured in thousands of glucose units, the significance of periodate oxidation results may be suspect.

Enzymic studies on amylose produced conclusive proof of its linear nature. Meyer et al (1941), found that the polysaccharide was completely degraded by β-amylase. Now as this enzyme is known to degrade chains of α-1:4-glucose units from the non-reducing end of the molecule by the stepwise removal of maltose, then the only possible structure for amylose appears to be an unmodified chain of α-1:4-linked anhydroglucose units. However Peat, Whelan and Pirt (1952) found that they could fractionate soya-bean β-amylase to yield (1) a pure β-amylase which did not completely hydrolyse potato amylose and (2) a second enzyme (Z-enzyme), which rendered the amylose susceptible to complete hydrolysis by the pure β-amylase. Banks and Greenwood (1960) showed that this enzyme (2) was a modified α-amylase. Thus the concept of amylose as a linear chain of anhydroglucose units linked by α-1:4-glycosidic linkages was no longer tenable.

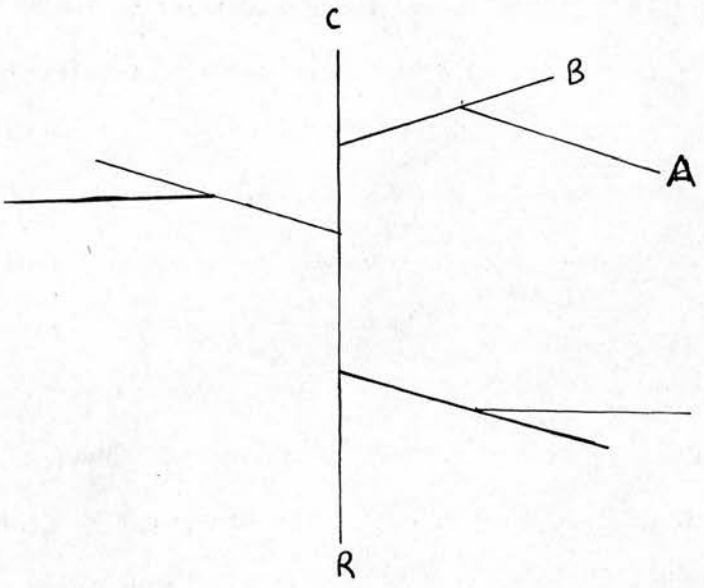
Cowie and Greenwood (1957b) and Arbuckle and Greenwood (1958) were able to confirm the above result and also to subfractionate amylose. They obtained sub-fractions which were linear in nature i.e. were completely converted into maltose by the action of the purified β -amylase, as well as fractions which were incompletely hydrolysed i.e. which contained a barrier to the enzyme action. This led to the postulate of two types of amylose in the starch granule; one probably containing a randomly-situated barrier and hence having a β -amylolysis limit of 50% conversion into maltose; the other being completely linear. Although it is now generally recognised that there is present in some amyloses, modifications of the molecule which produce a barrier to β -amylolysis, no widespread agreement exists as to the nature of this modification.

The Structures of Amylopectin and Glycogen. - The structure of amylopectin and glycogen is similar, although amylopectin is found basically associated with amylose in starch, while glycogen is the reserve polysaccharide of animals. There is probably a much closer relationship between the two molecules than has hitherto been supposed. (see p. 26)

Fine Structure of Amylopectin and Glycogen. - Methylation and periodate-oxidation studies on a number of amylopectins have indicated that the chain-length may vary from 18-27 anhydroglucose units. The corresponding range for glycogen is 12-18 anhydroglucose units.

In both polysaccharides, the main linkage is α — 1:4, with a small number of α -1:6 branch-points. Montgomery et al in 1949 isolated iso-maltose from various amylopectins after enzymic hydrolysis, thus indicating the presence of α -1:6 linkages. Their presence was finally proved by

Thomson and Wolfrom (1951) who obtained isomaltose and panose in the partial acid hydrolysis of waxy maize amylopectin. Periodate oxidation studies have also indicated the possible presence of 1:3- and even 1:2-linkages in amylopectin. Peat, Thomas and Whelan (1952) suggested that both amylopectin and glycogen contain three basic types of unit chain, each of which is linear and composed of α -1:4-glucose residues viz. A-chain linked to the molecule only by a 1:6-linkage to an adjacent chain; B -chain to which one or more A -chain is attached and which is itself attached by a 1:6-linkage from the reducing group to an adjacent chain; C-chain to which other chains are attached and which carries a free reducing group (R)



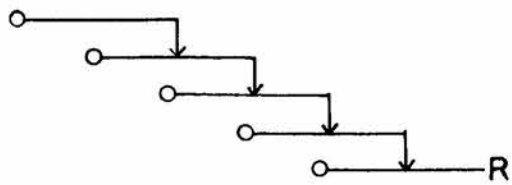
Amylopectin consists of a number of short chains of α -1:4-linked glucose units joined to each other by α -1:6-linkages. There are obviously several ways in which these unit chains may be arranged about each other and the

three principle arrangements are shown in Figure 1.1. Haworth, Hirst and Isherwood (1937) proposed the "laminated structure", which was consistent with methylation studies but was not intended to be a complete representation of the molecule. Straudinger and Husemann's (1937) "herring-bone" structure was derived by a comparison of the viscosity of amylopectin with that of cellulose of the same molecular weight. It was, however, only by enzymic studies that a more accurate representation of the amylopectin molecule was achieved. Meyer et al.(1940) showed that only ca. 50% of the amylopectin molecule was converted to maltose by β-amylase. This led to Meyer's postulated structure which was designed to accommodate both molecular weight and enzymic evidence. Three arguments favouring the Meyer formula have been advanced. Meyer and Bernfeld (1940) prepared the β-limit dextrin of maize amylopectin and subjected this to the action of a yeast preparation known to have maltose splitting activity which it was claimed could also hydrolyse the α-1:6-linkages. By this means 7% (as original amylopectin) of the dextrin was converted into glucose and the dextrin became susceptible to β - amylase action, 9.5% maltose (as original amylopectin) being liberated. It was argued that this incomplete hydrolysis by β-amylase supported Meyer's structure and showed the inaccuracy of the Staudinger structure which would have been completely freed from 1:6 bonds by the action of the yeast enzyme, and which would therefore have given a product which would have been completely hydrolysed by β-amylase.

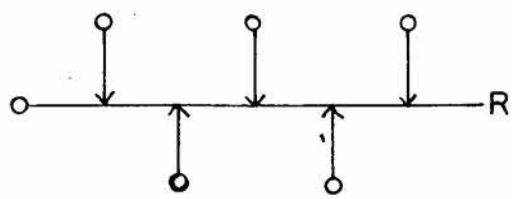
The second line of evidence in favour of Meyer's structure was produced by Peat, Whelan and Thomas (1952a). Again the experiments were concerned with the β-limit dextrin, in this case from waxy maize starch. The dextrin was submitted to the action of R-enzyme which hydrolyses the

FIG. 1·1

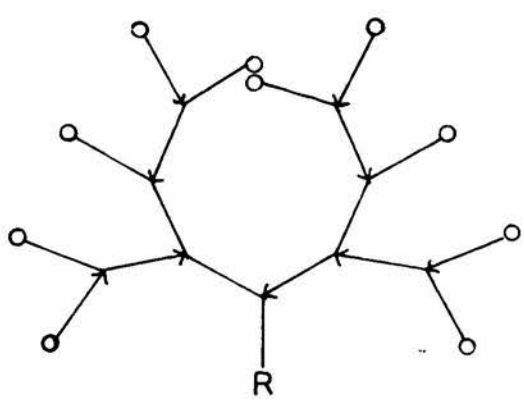
(a) Haworth



(b) Staudinger



(c) Meyer



R Reducing end group

o Non reducing end group

↓ α-1:6 link

1:6-bonds but not the 1:4-bonds. Fractionation of the products showed that maltose and maltotriose were the smallest molecules present. Malto-tetraose and pentaose were absent. Thereafter, a continuous series of polymers from maltohexose upwards was detected. The yield of maltose and maltotriose amounted to 12.8% by weight of the dextrin. It was argued that the maltose and maltotriose originated only from the A-chains. Now Haworths structure contains only one A chain and the probable yield of maltose and maltotriose from such a molecule would be less than 0.1% if the molecular D.P. of the β-dextrin were about 3,000. The Staudinger structure contains only one C chain and the remainder are A chains. In order to satisfy the requirements that the degree of β-amylolysis is 52% and that the unit chain-length of the amylopectin is 24.4, the β-dextran molecule would have to yield 20.8% of its weight as maltose and maltotriose. The evidence therefore favoured the Meyer structure which implies a proportion of A-chains intermediate between those in the Haworth and Staudinger structures.

The third approach to this problem was made by Larner et al (1952) using the enzyme amylo-1:6-glucosidase in conjunction with rabbit muscle phosphorylase. In the presence of inorganic phosphate, the phosphorylase converted 44.0% of wheat amylopectin and 52.9% of maize amylopectin into glucose 1-phosphate by an endwise, β-amylase like, progression along the outer chains of the molecule. The undegraded portion of each amylopectin was a resistant limit-dextrin in which an A-chain had been reduced to a single α-glucose unit attached to the molecule by a 1:6-link, while the outer portion of the B-chain was reduced to five glucose units in length

(Cori and Larner 1951). The separate action of the amylo-1:6-glucosidase then removed all the degraded A chains in the form of glucose by hydrolysis of the 1:6-linkages, the weight of glucose in each case being about 5-7% of the original phosphorylase limit dextrin. Having inactivated the glucosidase, the phosphorylase was again allowed to act and a second limit dextrin was formed, again susceptible to glucosidase action, but this time yielding a smaller percentage of glucose. Larner et al concluded that these results could only be reconciled with the Meyer structure. Beckmann (1953) has however shown that these results may also suggest the presence of more than one type of structure but this does not alter the principle conclusion of the work of Larner et al (1952), that the dominant feature of amylopectin is dichotomous branching.

The β-amylolysis limits of amylopectins, show a wide variation from 48-58% (Greenwood 1960). This limit is a measure of the external chain-length, as the enzyme action ceases when the branch-point is reached and the unattacked dextrin contains stubs of A-chains which are two or three units long depending on whether the original external chains had an odd or even number of units.

Posternak (1935) showed the presence of esterified phosphate at carbon atom six in tuber- and root-starches. Schoch (1942) found that the phosphorous was associated basically with the amylopectin fraction, and may be responsible for the low β-amylolysis limit of the polysaccharide.

Glycogen is very similar in basic structure to amylopectin. Its structure has been deduced by experiments similar to those detailed above

for amylopectin (for review, see Whelan, 1958). It was shown to consist of a number of unit-chains, each containing 10-14 glucose residues joined by α -1:4 bonds, with α -1:6 inter-chain linkages. The degree of branching in glycogen is twice that recorded for amylopectin and like amylopectin, glycogen possesses dichotomous branching.

Hydrodynamic behaviour of amylopectin and glycogen. - Although glycogen and amylopectin are similar in chemical structure, their solution properties are quite different.

The limiting viscosity number of glycogen is small ca. 10 (Bryce, Greenwood and Jones, 1958), while that of amylopectin is quite large ca. 150 (Cowie, and Greenwood, 1957). The small limiting viscosity number for glycogen suggests that the molecule is spherical and in agreement with this the concentration dependence of its sedimentation coefficient is small. On the other hand, amylopectin exhibits a very large concentration dependence during sedimentation. Two explanations may be advanced to account for this behaviour (1) the amylopectin molecules themselves may be spherical but their hydrodynamic behaviour may be governed by the entanglement of the external chains of several molecules, or (2) the molecular shape may be quite different from that of glycogen. Little evidence exists to support either of these explanations, although the crystalline aggregate or "micelle" structure of the starch granule proposed by Meyer and Bernfeld (1940) would probably require a two-dimensional disc-like structure (Greenwood 1956) rather than a three-dimensional spherical structure.

Ib. The Chemistry and Biology of the Starch Granule.

"Starch is composed of minute cells or granules so small that it takes three-quarters of a trillion to make 1lb. of cornstarch"

(Schoch 1940)

The starch granule has been the subject of a tremendous amount of research. During the period 1820-1900, it was a most popular subject for microscopic study. In 1836, Poggendorf wrote that "the starch granule was one of the most studied, and least understood of all substances." Recently Badenhuizen (1959), after an extensive review of starch granule research, said that it was apparent that little more was known to-day than 100 years ago. In short, the starch granule, with all its peculiar characteristics, is still an intriguing unknown. Many questions yet remain unanswered e.g. where and how is the granule formed?, what is the molecular architecture of the granule?, how are amylose and amylopectin produced in the granule?. With regard to most of those questions there has been considerable speculation much of which has subsequently proved to be erroneous.

Origin of the starch granule.

Most investigators believe that starch granules originate in the amyloplasts. Badenhuizen (1939), Frey-Wyssling (1936), Yasui (1949) and O'Brien (1951) have all independantly come to this conlcusion. Weier (1936) and Guilliermond (1941) consider that the amyloplasts are derived from the chrondriosomes, and Badenhuizen (1939) considers that the chrondriosomes are filiform. Duvick (1953) and Shaw (1954) both came

FIG. 1-2

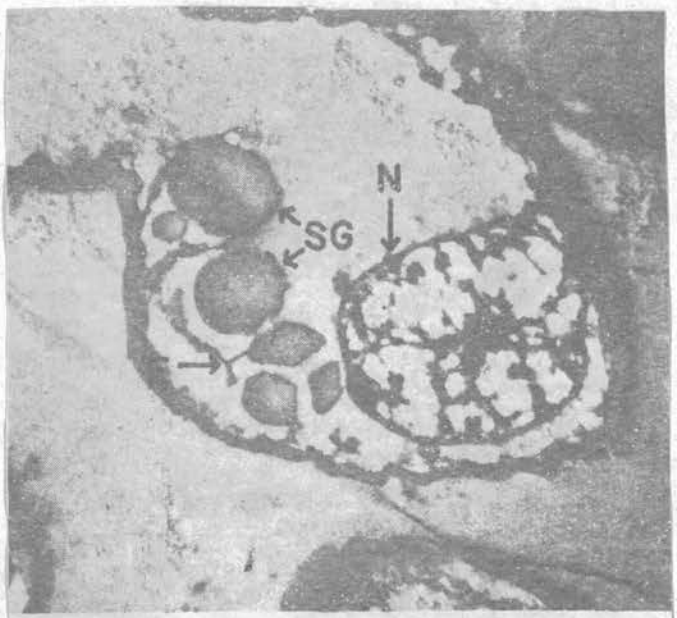


Figure 1. Cross section of kernel 4 days after pollination

Heavy line is cell wall
SG. Starch granule
F. Knobbed filaments
N. Nucleus

a.

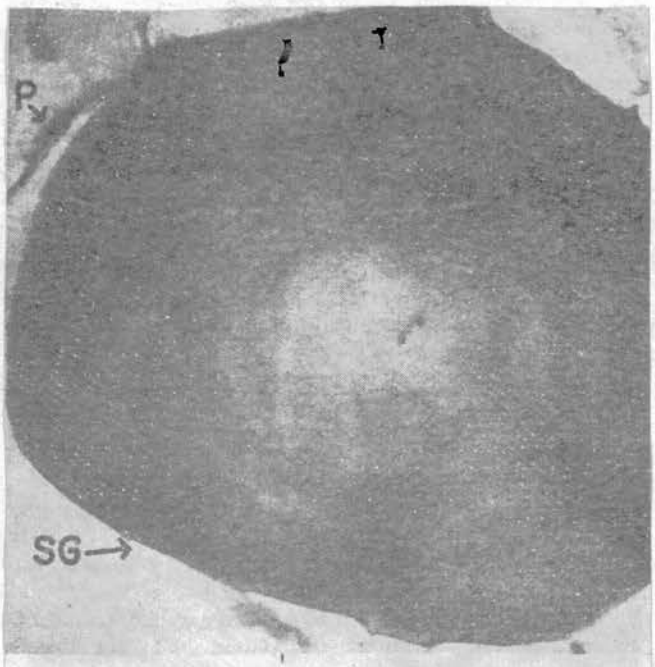


Figure 3. Section at 18 days, 25,000×

SG. Starch granule
P. Plastid membrane

b.

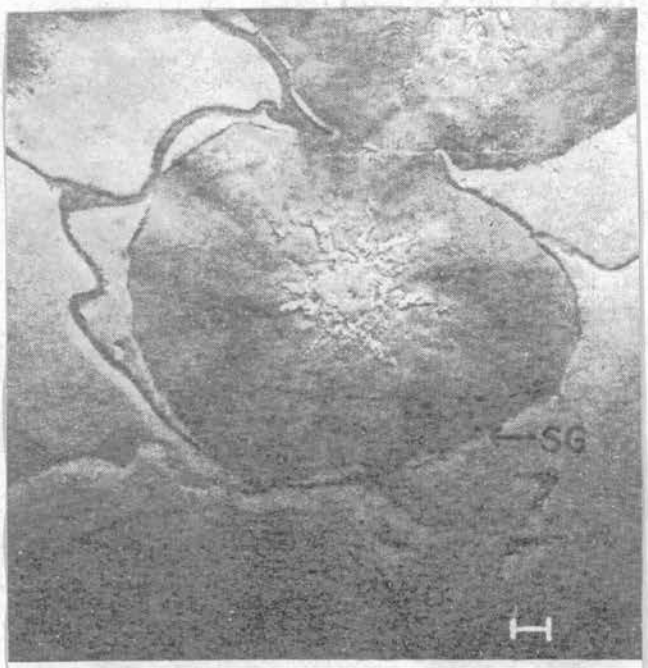


Figure 7. Section at 72 days

SG. Starch granule

c.

Figure 8. Horny section at 70 days

SG. Starch granule

d.

to the conclusion that the amyloplasts contain phosphorylase, which is an essential enzyme required in the synthesis of starch. Duvick (1953) also described large-knobbed filaments (plastids) which give rise to starch granules. The largest knob in any given cell is the first to form starch but not all such knobs form starch.

Figure 1.2. shows photomicrographs taken by Whistler and Thornburg (1957) which show clearly the various stages in the formation of a granule of corn starch. Those workers observed starch granules in corn endosperms after as little as 4 days after polination (Fig. 1.2a). The measuring bar on each of the photographs is equal to 1 micron and as can be seen, the granules increase in size as they mature. In Fig. 1.2a. and 1.2b., the young granules are surrounded, by a membrane, which is undoubtedly the membrane of the amyloplasts. As the granules mature they completely fill the amyloplast and often rupture it. Fragments of the amyloplasts may be seen attached to granules at all stages of maturity beyond 12 days after polination (Fig. 1.2c). This rupturing of the amyloplast membrane has been used to explain the presence of a lamellaed structure in some starch granules and will be dealt with later.

<u>The physical structure of the starch granule</u>.

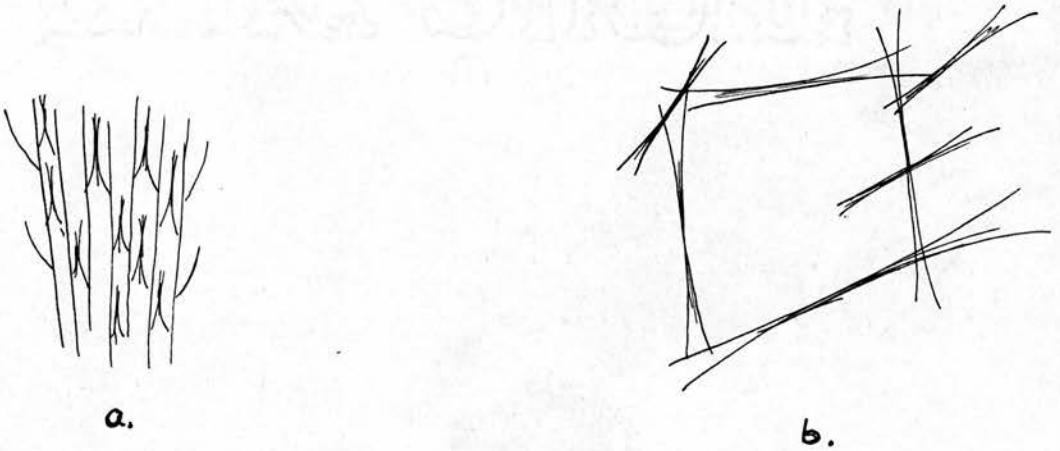
Microscopic examination has thrown some light on the physical structure of the starch granule. The first microscopic investigation of starch was made by Leeuwenhoek in 1716. He concluded that the granule consisted of a kernal or centre which "is fit for nourishment" and an outer insoluble non nutritive envelope. One difficulty encountered in starch granule study is caused by the fact that starch granules absorb water and become transparent

This is coupled with a slight swelling effect. Measurements of longitudinal-axis swelling have shown that potato granules increase by 47%, and pea granules decrease by 2%, while the short axis show an increase of 29 and 35% respectively (Hansenn, Dodt and Niemann, 1953). Microscopic observation of those swollen granules have shown many structural features. Frey-Wyssling and Badenhuizen (1939) gave warning that the features thus shown are artefacts which do not necessarily reflect the original structure. Other workers, Sponsler (1922) and Hanssen et al (1953) believe that, although the structural features are invisible before swelling, the features revealed by the water treatment pre-exist in the granules. The swollen granule would seem to be a spherocrystal made up of fiber-like crystals, which orientate both radially and concentrically. As the granule swells in water, a layered or lamellaed structure become visible. According to Badenhuizen (1938) the consistency of the layers change from hard to soft when the granules are cut by a micro-knife. There would seem to be a reasonably simple explanation for such a layered structure, although as will be seen later speculation has led to some unusual ideas regarding this phenomenon. If the granules are made to undergo further swelling at higher temperatures, other structures appear which are referred to as "blocklets". Here the layers are broken regularly into small parts. These blocklets may again be pre-formed structures of the native granule but no coroborating evidence of this has as yet been produced. If the granule is heated further to just below its gelatinization temperature, a liquid centre is produced which seems to be enclosed by a thin skin.

This fact and the light defraction patterns at the periphery has been convincing proof to many workers, of the presence of an external membrane.

It is obvious from the literature that there has been considerable speculation as to the nature of the molecular architecture within the starch granule, but K.H. Meyer (1942) without much experimental evidence formulated the concept of granule structure which is most widely accepted to-day. According to his theory, the intermixed linear and branched starch molecules are arranged in the granule in a radial fashion as shown in Fig. 1.3a.

Figure 1.3.



a. b.

Wherever linear sections of either branched or linear molecules parallel one another, hydrogen bonding forces pull the chains together

into associated crystalline bundles or "micelles". A long linear-chain may conceivably pass through a number of such micellar areas; or the outer fringe branches of a branched molecule may participate in several separate micelles. Hence these crystalline areas hold the granule together. This type of structural system can be used to explain several of the characteristics of starch e.g. swelling and leaching properties. In the spaces between the micelles the chains and branches are more disordered and hence less densely packed. When an aqueous suspension of a granular starch is heated it is presumed that these amorphous areas undergo progressive hydration and swelling. Eventually the expanded network[1.3b] is produced held together by the persistant and intact micelles. It is this network which gives the granule its elastic character and which is responsible for most of the viscous properties. These micellar regions can be dispersed by boiling. The variations in the micellar patterns of various starches, explain the differences in their properties. Pea starch and potato starch are, for example, extremely different from one another, both in physical and chemical properties. Potato starch granules gelatinize at 61°C and are easily dispersed in boiling water, while pea starch granules do not gelatinize even on boiling water and cannot be dispersed without pretreatment with caustic soda or liquid ammonia. Aqueous leaching of these defatted starches gives rise to varying amount of unmodified amylose. Potato gives about 40% while pea similarly leached gives only 1.5% linear amylose. All these

differences can be explained on the basis of this "micellar theory". It is obvious that the bonding forces within the granule will influence the swelling of the granule. Hence a starch which is resistant towards swelling must be a highly associated starch, i.e. it should have an extensive and strongly bonded micellar structure. Hence by this postulation, it would follow that pea starch granules have a more intense micellar structure. If this is true it is obvious that aqueous leaching of such a granule will give little linear amylose. Linear amylose is presumably obtained from the amorphous regeons of the granule. There will obviously be a considerably greater amorphous area in potato granules than in pea granules and hence the linear amylose produced on leaching will be approximately proportional to this amorphous area present.

This micellar theory assumes that the linear and branched molecules are bonded together by hydrogen bonding. Many starches have however a high moisture-content and it is conceivable that bonding may take place through hydrated (H-O-H) bridges rather than by this direct association.

The composition and formation of starch in the starch granule. - The study of the starch present in the granule must be considered both from a biological and a chemical viewpoint. In forwarding any plausible theory of starch formation, biological and chemical rules must be obeyed. It is necessary to continually analyse the result of both sciences and neglect of one or the other has in the past led to extreme confusion and the development of several erroneous theories.

Before 1940, microscopic observations could only show an apparent

chemical homogeneity in the structure of starch. In 1942, the presence of linear molecules designated the name amylose and branched molecules called amylopectin was demonstrated very convincingly by T.J. Schoch. However this "multiple" concept is not universally accepted. Whilst most workers are agreed on the properties and structures of the separated starch components, not everyone is agreed that they exist as such in the native starch granule. Several hypotheses, collectively termed the "unitary concept", picture the starch grain as consisting of amylose and amylopectin molecules linked to each other by bonds which are split when the granule is dispersed in hot water. From this point of view, amylose and amylopectin are regarded as artefacts, and starch is looked upon as one big molecule. Although the "multiple concept" commands a much wider support than the "unitary concept", the arguments favouring the latter viewpoint merit careful study and further investigation. One argument in its favour is that maize starch dispersed in cold alkali or boiled in oxygen-free alkali and then in each case neutralised, cannot be fractionated by butanol. Separation of the butanol-amylose complex is only possible after heating the neutral solution above 60° (Bauer and Pacsu, 1953). However a much stronger argument for the pre-existence of the two separate components exists. It is known that starch granules grow by apposition. This was proved for potato starch by Badenhuizen and Dutton in 1956 and by Badenhuizen and Gaffin in 1959. As this means that layers are deposited on the outside, often at irregular intervals, it is difficult to imagine how the molecular chains of the new

layer could link up with those of the old layer, especially as the latter contains starch in the retrograded condition, when it cannot fulfil the function of primer molecules needed for synthesis by phosphorylase (Whelan and Bailey 1954). Further the "unitary concept" would make it even more difficult to explain why there is always found a practically constant genetically fixed percentage of linear molecules in a particular starch, than it is now (see Table 1.2)

Table 1.2.

Plant Source	% Amylose			
Banana	16.8 (a)	19 (b)	16.4 (c)	
Smooth Pea	35 (d)	35.4 (a)	34-37 (b)	35 (c)
Wrinkled Pea	66 (d)	64.7 (a)	61-70 (b)	66 (c)
Broad Bean	25 (b)	23 (c)		
Potato	22.9 (e)	21 (f)	20-25 (b)	22 (c)
Iris	27 (f)	27-29 (b)	26 (c)	
Parsnip	11.1 (g)	11 (c)		
Amylomaize	54 (i)	52 (c)		
Rubber seed	20 (j)	19 (c)		
Barley	19 (h)	22 (f)	24-27 (b)	22 (c)

(a) Larson and Gillis (1953), (b) Deatherage (1955), (c) Greenwood and Thomson (1960), (d) Potter et al (1953), (e) Doremus et al (1951), (f) Anderson et al (1955), (g) Anderson et al (1956), (h) McWilliam

and Percival (1952), (i) Senti (1960), (j) Greenwood and Robertson (1954).

The synthesis of Starch in the plant. - Little is known of the enzyme system involved in starch synthesis in the plant. The synthesis of starch in Nature usually involves the production of both amylose and amylopectin. Bates, French and Rundle (1943) pointed out that amylose and amylopectin cannot be produced at the same time under equilibrium conditions. Experiments in vitro using phosphorylase and the branching enzyme Q-enzyme, have shown that only the branched component is synthesised and that the degree of branching for the most part depends on the ratio of phosphorylase activity to the branching enzyme activity. This fact means that a specialised enzymic system or enzymic equilibrium must be present before starch can be produced.

Many theories for this synthesis have been postulated. Some are quite logical and possible, while others considered in the light of biology and chemistry are obviously wrong. From the literature, it can be concluded that the contents of the starch granule are synthesised in the plant, (1) at the same time by phosphorylase and Q-enzyme but under non-equilibrium conditions (2) at different periods of the day i.e. amylose synthesis predominating during the day and amylopectin synthesis during the night (3) from pseudo-amylose (Bourne and Peat, 1945) (4) in layers separated from each other by membranes (5) in different parts of the amyloplasts (Whelan, 1958) (6) by a hitherto undiscovered enzymic reaction (Erlander, 1958). All these theories have their merits and demerits, but at least two have distinct possibilities although it is

difficult to see how proof of any of them can be produced.

1. The synthesis of starch by enzymes under non-equilibrium conditions. - There is at present no reason to outrule this mechanism for the production of starch by the combined action of phosphorylase and Q-enzyme, but it is difficult to imagine restrictive forces which could prevent an equilibrium distribution of the branched enzyme or its inhibitor.

2. Synthesis of amylose and amylopectin at different periods of the day. - An old untried theory was held that amylose was produced in the plant during the day and amylopectin during the night. Since the activity of phosphorylase remains essentially constant throughout the day, a fact verified by Badenhuizen and Malkin in 1955, one can postulate that the branching enzyme is inactivated during part of the day in order that amylose may be produced. Erlander (1958) collected sweet and dent corn ears at 12 hour intervals and found that amylose and amylopectin are produced at the same time of the day and indeed the largest production of both components occurred during the night.

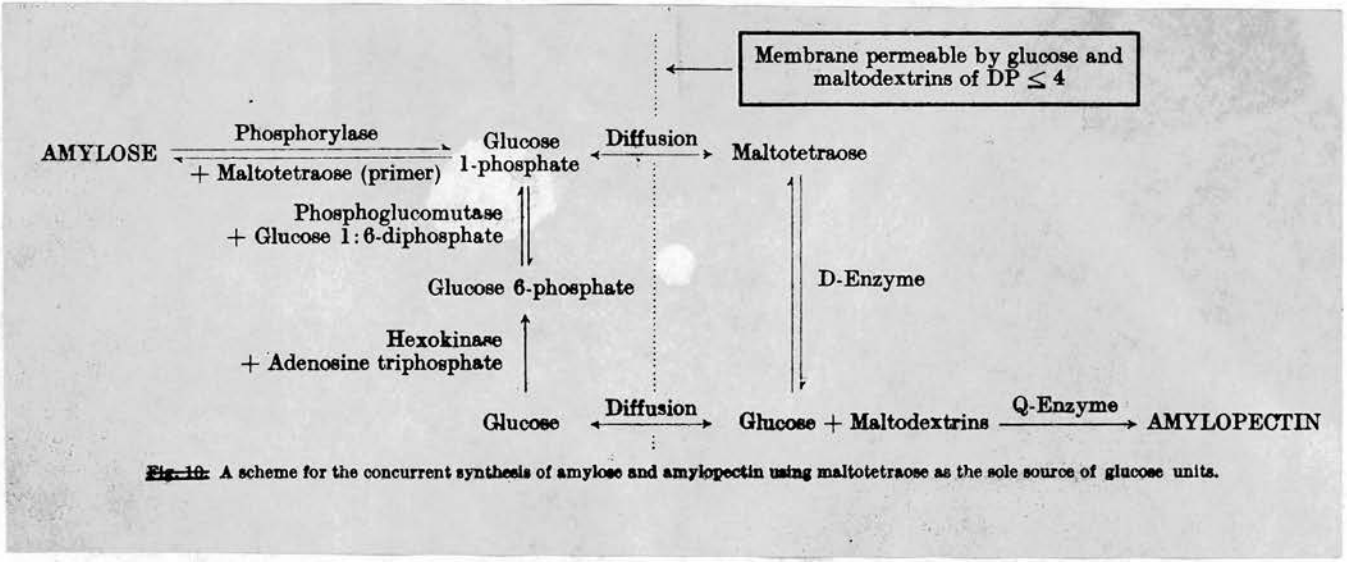
3. Synthesis of amylose and amylopectin from pseudo-amylose. - In order to explain how starch synthesising cells can produce both linear and branched polymers - presumably at the same time - Bourne and Peat (1945) proposed a "pseudo-amylose" intermediate. This intermediate had an average length equal to the average chain-length of the amylopectin molecule and was capable of being converted into either amylose by further use of phosphorylase or into amylopectin by means of an enzyme which would link the pseudo-amylose chains together through 1:6-linkages. Hobson et al

in 1951 studied the branching enzyme and showed that the amylose-type substrate had to be at least 42 units long before it is attacked by Q-enzyme thus showing that the pseudo-amylose proposed mechanism is unlikely.

4. Synthesis of amylose and amylopectin in alternating layers. - This theory was the natural successor to the theory of amylose and amylopectin formation at different periods of the day. Badenhuizen (1946) studied the layers found in starch granules and found that they changed alternately from hard to soft when cut by a micro-knife. Iodine staining of the granules show that the linear and branched materials are completely intermixed and that no separate layers of amylose and amylopectin actually exist in the granule. This type of theory which requires the presence in the starch granule of some kind of obstacle to the free diffusion of the enzymes has however led Whelan to forwarding a new and plausible starch synthesising mechanism involving amembrane system (Whelan, 1958).

5. Synthesis of amylose and amylopectin in different parts of the Amyloplasts. - The in vitro action of the starch-synthesising enzymes offers no ready explanation as to how amylose and amylopectin are concurrently synthesised in the same starch granule. Bates, French and Rundle (1943) showed that the action of phosphorylase and Q-enzyme concurrently, on glucose-1-phosphate and primer, gives only a branched polysaccharide, while Barker, Bourne, Peat and Wilkinson (1950) obtained in the same way, a graded series of polysaccharides whose degree of branching was related to the varying amounts of

FIG 1·4



~~Fig. 10.~~ A scheme for the concurrent synthesis of amylose and amylopectin using maltotetraose as the sole source of glucose units.

Synthesis No. 5

phosphorylase and Q-enzyme in the particular digest.

Whelan in 1958 forwarded a new theory of starch synthesis. He supposed that within the space to be filled with synthesised starch, there may be some obstacle which prevented free diffusion of the enzymes. This obstacle, e.g. a membrane, makes possible the formulation of a hypothesis to explain how the concurrent synthesis of amylose and amylopectin could occur.

This system requires two compartments separated by a semi-permeable membrane (see Fig. 1.4.) capable of passing molecules in size up to, say, maltotetraose. In one compartment are D and Q-enzyme and in the other hexokinase (+ adenosine triphosphate), phosphoglucomutase (+ glucose 1:6-diphosphate) and phosphorylase. The source of glucose units from which the amylose and the amylopectin are ultimately synthesised is maltotetraose or any maltodextrin from maltotriose upwards.

According to Whelan's hypothesis the first enzyme to exert its action will be the D-enzyme which causes a disproportionation of the maltotetraose into other maltodextrins and glucose. This glucose passes through the membrane and is converted into glucose-1-phosphate (G.1-P) by the hexokinase-phosphoglucomutase system. Synthesis of amylose from G.1-P and maltotetraose will ensue and as the growing amylose chains cannot diffuse through the membrane, they cannot be attacked by the branching enzyme. Meanwhile in the D + Q-enzyme compartment the removal of glucose, by disturbing the equilibrium in the D- catalysed reaction will cause a further production of glucose with a consequent increase in the average

length of the maltodextrins. Once these become sufficiently long (about 40 units) to be acted upon by Q-enzyme the synthesis of amylopectin will commence and in the same way as the synthesised amylose was protected from Q-enzyme by the membrane so will the amylopectin also be unable to pass through the membrane into the phosphorylase compartment.

Many arguments have been forwarded, both supporting and opposing Whelan's hypothesis. Consideration of this scheme will show that the net result will be the incorporation of three glucose units of maltotetraose into amylopectin and one glucose unit, liberated by the action of D-enzyme from the reducing-end of the molecule, into amylose. The resulting weight ratio, amylopectin:amylose of 3:1 corresponds to the most usual ratio found in natural starches. On this hypothesis, waxy maize starch granules which contain only amylopectin must be produced in an amyloplast where no membrane system exists.

This scheme requires however that the amylose and amylopectin are produced in different parts of the amyloplast. The two components must therefore diffuse from their origin to a common centre of the cell in order to form a starch granule. If the amylose and amylopectin can migrate from their birth place, there is every reason to believe that the enzymes themselves should also be able to migrate to different parts of the cell in the same way.

6. Synthesis of starch by a hitherto undiscovered enzymic system. - The problem of concurrent amylose - amylopectin synthesis was further complicated by the discovery by Hassid and McCready (1941) of a glycogen-

type polysaccharide in sweet-corn endosperm. It is difficult to imagine the synthesis of highly branched glycogen, lesser branched amylopectin and unbranched amylose in any amyloplast by any of the enzymic methods which have been considered in theories 1-5. Indeed according to Erlander (1958), all these methods are unable to explain the presence of a glycogen-type polysaccharide. Erlander therefore proposed a new mechanism for the synthesis of starch. He supports the idea first forwarded by Wolf et al (1948), who had found that sweet corn granules lie embedded within globules of glycogen. They had therefore suggested that glycogen may act as an intermediate in starch-synthesis. Erlander proposed a mechanism for such a reaction which not only explained the formation of starch, but also the production of the granular rings.

Any proposed mechanism for the synthesis of amylose from glycogen must account for the large size of the amylose molecule. As can be easily seen complete scission of all 1:6-linkages in glycogen would yield only small 1:4-linked linear chains. The following mechanism was therefore proposed by Erlander:-

$$\text{glycogen} + \text{DE-P} \longrightarrow \text{DE-Gx} + \text{amylopectin-P}$$

$$\text{DE-Gx} + \text{Gn} \rightleftharpoons \text{G(n + x)} + \text{DE}$$

$$\text{DE} + \text{ATP} \longrightarrow \text{DE-P} + \text{ADP}$$

or the overall mechanism will be

$$\text{glycogen} + \text{Gn} \xrightarrow[\text{DE - enzyme}]{\text{ATP} \quad \text{ADP}} \text{amylopectin - P} + \text{G(n + x)}.$$

Erlander's mechanism is shown pictorially in Fig. 1.5 for the debranching of the ideal Meyer model. Here we see that the phosphorylated debranching enzyme DE-P, transfers its phosphate group (P) to the branch point of the glycogen molecule and simultaneously becomes linked with the aldehydic group of the branch. The complex DE-Gx, between the debranching enzyme (DE) and the debranched chain Gx containing x glucose units, is similar to that enzyme complex involved in the branching of glycogen (Barker and Bourne, 1953). The DE-Gx complex formed can then transfer its chain to the non-reducing end of the receptor group Gn. This theoretical debranching enzyme (DE) can then be phosphorylated by some source such as adenosine triphosphate and can attack another glycogen molecule. The resulting linear G(n + x) molecule, now having (n + x) glucose units can again act as an acceptor and the long linear chains of amylose formed can crystallize out of the medium before being attacked by the branching enzyme. According to Erlander the partially debranched glycogen (amylopectin) may also crystallize out and the variation in the rate of crystallization causes the production of the granular rings.

Figure 1.5.

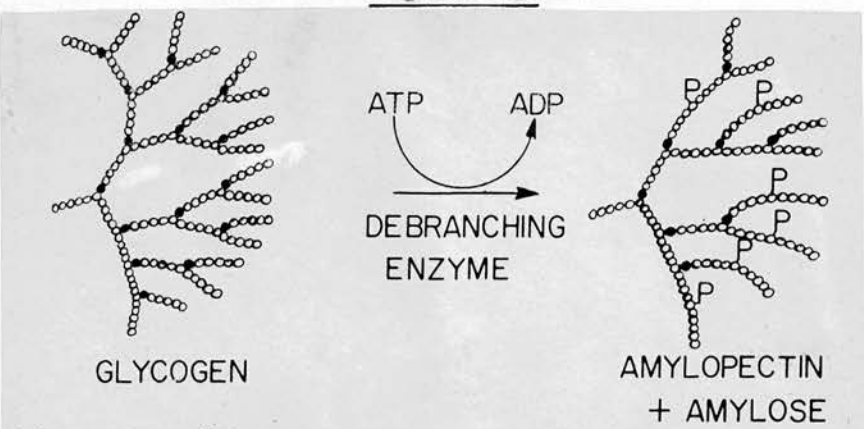


Fig. 1.5 The synthesis of starch from glycogen (see text). Some outer and inner branches of the ideal MEYER model for glycogen are removed by the phosphorylated debranching enzyme to give a simplified illustration of the proposed debranching mechanism. Each circle represents a glucose unit. The black circles are those glucose units connected by 1,6-linkages to the branch points. The white circles are connected through 1,4-linkages. (P) represents phosphate.

It is obvious from this mechanism that the glycogen molecule may be attacked more than once by the debranching enzyme. This debranching enzyme may display the phenomena of steric hyndrance in its attack on the most susceptible branch points of the glycogen molecules. Inner branches, which are exposed by the removal of the surrounding exterior branches, may be more susceptible to attack by the debranching enzyme than remaining external branches. This would explain the longer internal and external branches found in amylopectin. The debranching reaction itself must also be irreversible. If it were reversible the Gx molecules produced by the debranching enzyme attack would most probably attach themselves to the many available 6-positions. The amylose produced by this mechanism G(n + x), would most likely reach an optimum chain length as a receptor group and this optimum would depend upon the average branch length of the glycogen precursor and the number of receptor groups available to the DE-Gx complex. The original receptor group Gn must have an available non-reducing glucose group and could be of any size, glucose or maltose being the lower limit.

This theory also gives an explanation for the formation of waxy-type starches. If the debranching enzyme has a low activity due to the presence of an inhibitor or if there are few linear acceptor groups in the medium, then the amount of amylose produced will be extremely small.

Erlander's theory for the formation of starch from glycogen may not necessarily be the correct one but the actual synthesis of starch

from glycogen itself has more literature support than has any of the other theories of synthesis. Erlander's theory will explain the presence of glycogen in certain plant species and also the presence of organic phosphate in amylopectin but not in glycogen. There are however two basic reasons why Erlander's theory cannot be fully accepted. (1) There is no explanation for the presence or the action of Q-enzyme in this theory and (2) no debranching enzyme is known which will debranch glycogen to give an amylopectin molecule.

A detailed study of changes in the structure of amylose and amylopectin with increasing maturity of a plant, might give some check to Erlander's hypothesis, but until such a debranching enzyme is isolated and its action pattern studied, the theory must continue to be accepted with considerable reservation.

It is obvious, that although much work has been carried out by many workers on the structure of the starch granule and its contents, there is yet much to be done before their structure and synthesis are fully understood.

SECTION 2.

EXPERIMENTAL METHODS.

2a.

Determination of Glucose and Maltose concentrations.

The concentration of any glucan may be simply and conveniently determined by hydrolysis of an aliquot sample, followed by volumetric estimation of the reducing groups. The method of Somogyi (1945) using a copper reagent, is probably the most common method used for such an estimation, but a more recent method developed by Lampitt et al (1955) using the alkaline ferricyanide - ceric sulphate technique, has certain advantages. This is particularly true during the estimation of maltose in the presence of an iodine-staining polysaccharide, which occurs in the determination of the β -amylolysis limits of amylopectin and amylose. The copper reagent used in the Somogyi type estimation, releases iodine which may react with the polysaccharide, thus rendering the end-point of the thiosulphate - iodine titration susceptible to error. This difficulty is avoided by using the ceric sulphate method. Not only is there no interreaction between the reagent and the polysaccharide present, but repeated calibration of the reagent against glucose and maltose is unnecessary, because the calibration factor is directly proportional to the normality of the ceric sulphate. The latter is simply determined by titration against standard ferrous ammonium sulphate solution.

Experimental.

Estimation of glucan concentration:- Three known volumes of polysaccharide solution each containing up to 3 mg. of polysaccharide

were placed in Quickfit test-tubes, neutralised if alkaline, and 1 ml. of 3N-sulphuric acid added. The stoppered tubes were placed in a boiling water bath for 2 hours. At the end of this period, the tubes were cooled, and the contents neutralised with M-potassium hydroxide solution using bromo-cresol green as indicator.

Each solution was then diluted to ca. 10 ml. with distilled water. Sodium carbonate solution (2.5 ml., 0.2 M) and potassium ferricyanide solution (2.5 ml., 0.05 M) added and the tubes returned to the boiling water bath for 15 minutes. At the end of this time, they were cooled and sulphuric acid (5 ml., 5N) and two drops of xylene cyanol F.F. indicator added before titrating with 0.01N-ceric sulphate. The end point of this titration was indicated by a colour change from a sage green to a pale orange. Maltose was also estimated by this method directly without hydrolysis.

2b.

Potentiometric Iodine Titration.

In the study of starches it is essential, not only to know the composition of the starch but, after fractionation, to know the purity of the fractions obtained.

Bates, French and Rundle (1943) first introduced a potentiometric iodine titration method which was based on the greatly differing affinities of amylose and amylopectin for iodine. By this method, measurement of the potential difference between a calomel electrode and a bright platinum electrode in a starch-iodine solution permitted calculation of the equilibrium concentration of free iodine in the latter. The apparatus used in this work was developed by Anderson and Greenwood (1955) from that of Gilbert and Marriot (1948).

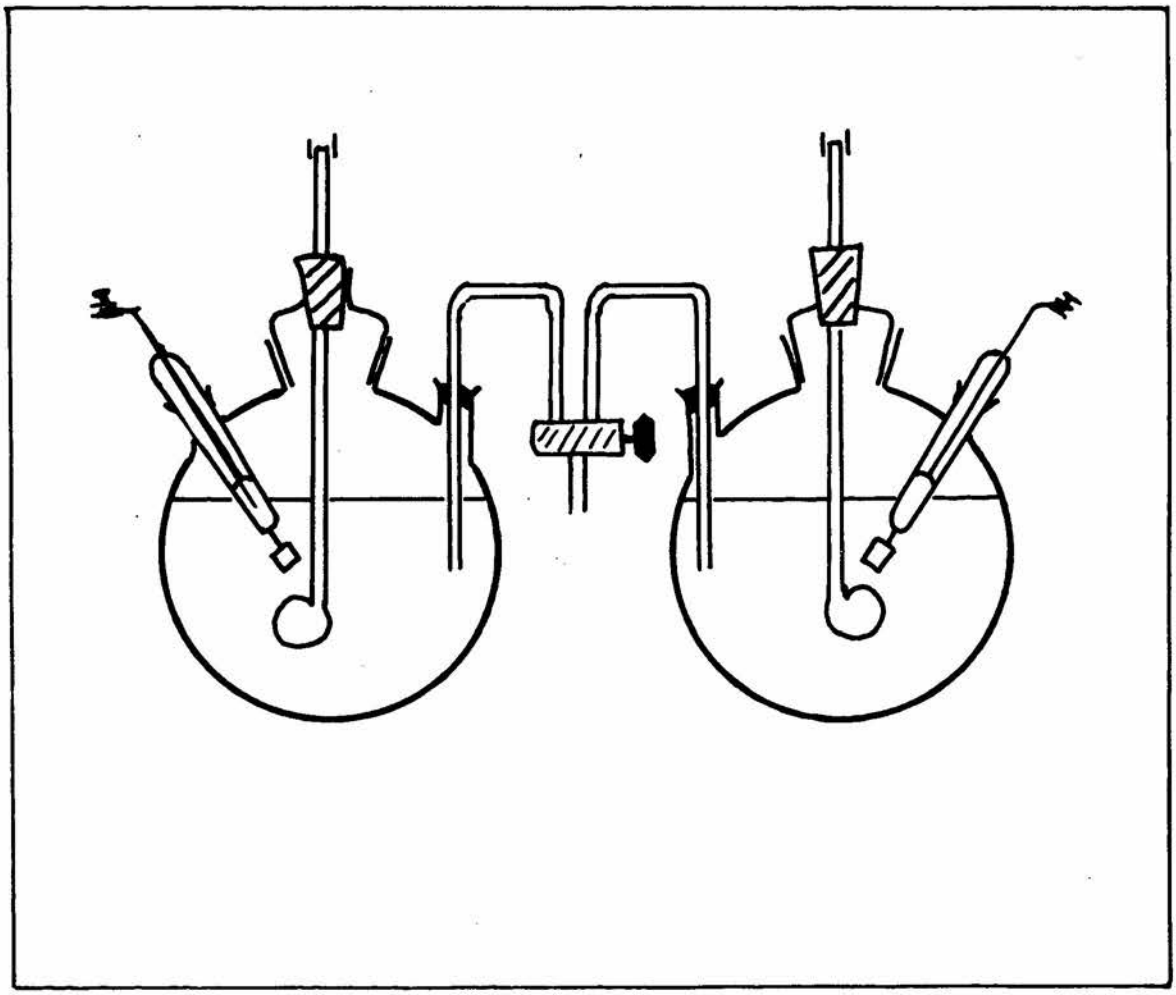
Apparatus. - A polysaccharide-iodide solution and a blank-iodide solution are contained in two opposing half-cells connected by a salt-bridge. The equilibrium free iodine concentration is measured by means of a null deflection method. This differential method is most sensitive at low free iodine concentrations. The apparatus had an electrometer of high sensitivity, with the necessary zero stability required for null-point determinations. This consisted of a matched pair of valves which acted as two of the resistors in a Wheatstone network, while the other two resistors were made the cathode loads for the valves. The fact that each valve functioned as a cathode follower ensures high stability. The electrometer

circuit was connected to the sample half-cell via a highly insulated two-way "make-before-break" switch so that the electrometer grid was never in open circuit. Solutions were stirred continuously in the titration cells, (1 ℓ. pyrex flasks) which had four necks fitted with ground-glass quickfit joints; three of these accommodated the stirrer, platinum electrode and salt bridge, while the fourth was for the addition of iodine (see Figure 2.1.). The electrodes were made of platinum foil, fused to platinum wires, which were each sealed through glass tubing containing a pool of mercury. No potential difference existed between these two electrodes when placed in the same electrolyte.

Preparation of sample solutions. - Approximately 3-5 mg. amylose, 25-30 mg. amylopectin or 10-12 mg. starch was placed in a weighing stick, inserted in a vacuum drier and left overnight. The weighing stick was then removed, stoppered and left to cool in a dessicator for 10 minutes. The sample was then weighed, transferred to a flask and dissolved in 0.2M potassium hydroxide (10 ml.). Before addition to the half cell the solution was brought to pH 5.85 by adding a predetermined volume of 0.4M-phosphoric acid. A blank containing the same volumes of potassium hydroxide and phosphoric acid was prepared and added to the other half-cell.

Conditions and procedure of a potentiometric titration. - Conditions for titration were: iodide , 0.01M; pH = 5.85, and temperature 20° (controlled thermostatically). The electrolyte solution contained M/10-potassium iodide (210 ml.); M/15 phosphate buffer

FIG.2·1



Cell Arrangement

Iodine Titration Apparatus

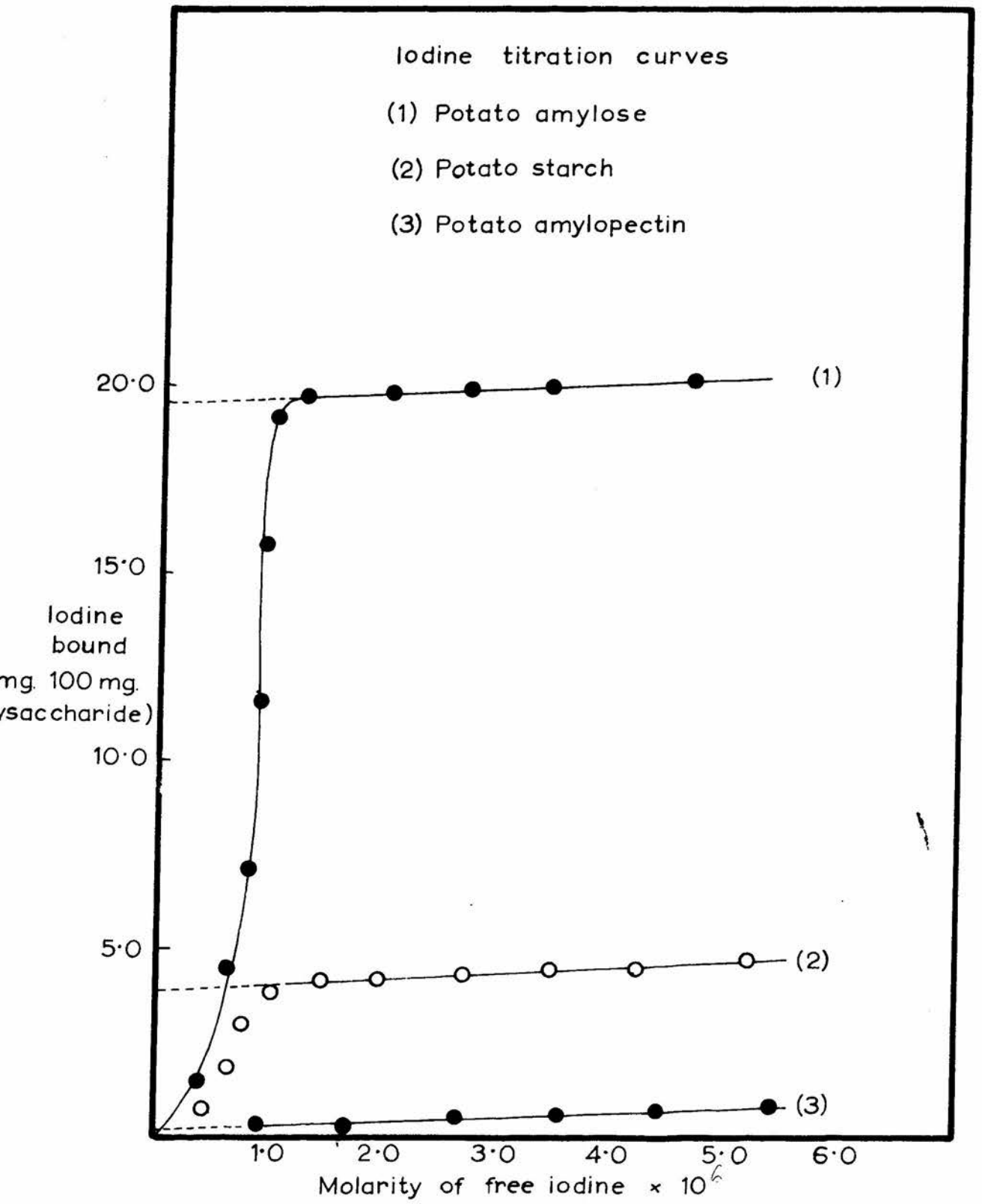
(15 ml, pH 5.85) and was made up to 2 litres with distilled water. Each half-cell contained 800 ml. of this solution which was allowed to reach temperature equilibrium before commencing titration.

The sample solution and blank were added to their respective half-cells and the flasks rinsed with water 25 ml, so that the final volume was approximately 840 mls. Before commencing iodine-iodide additions the circuit was checked to make sure there was no significant off-balance potential.

Small increments (0.1 ml) of 0.01 M iodine - potassium iodide solution were added to the solution cell by means of an "Agla" syringe. Time (2-5 minutes) was then allowed for equilibrium to be reached after each addition, before balancing the potential produced by the free iodine. This is accomplished by adding the same iodine-iodide solution to the control cell until the galvanometer deflection caused by the potential difference is zero. The difference between the volumes of iodine added to the half-cells gave the amount of iodine bound by the sample. A titration curve was thus constructed by plotting the iodine-bound (mg/100 mg of polysaccharide) against the total free iodine in the solution. (see Fig. 2.2.).

As can be seen from the curve, under these conditions amylose binds about 19.0% of its own weight of iodine. After saturation, the activity increased very slowly with increased free iodine concentration. Whereas Bates et al.(1943) took the "iodine affinity" of the amylose as the point of intersection of these two parts of the graph,

FIG. 2·2


Iodine titration curves
(1) Potato amylose
(2) Potato starch
(3) Potato amylopectin
20·0
15·0
10·0
5·0
Iodine bound
mg. 100 mg.
saccharide)
(1)
(2)
(3)
1·0
2·0
3·0
4·0
5·0
6·0
Molarity of free iodine × 10⁶

Greenwood *et al.* (1955) obtain the "iodine affinity" by extrapolation of the linear part of the graph to zero free iodine concentration. The maximum iodine binding power for pure amylose was found to be approximately 19.0% of bound iodine. Amylose percentages in other samples can be calculated on this basis.

2c

Enzymic Degradation.

Much of the knowledge gained in the last few years, with regard to the fine structure of amylose, amylopectin and glycogen has been obtained by critical enzymic studies on these substrates. Enzymes which will hydrolytically attack such substrates can be conveniently subdivided into at least five groups, these being (1) amyloglucosidases (2) debranching enzymes (3) phosphorylase (4) the endo-amylases and (5) the exo-amylases. The most important of these enzymes are the endo- and exo-amylase, represented by α and β -amylase respectively.

The recognition of the existence of these two types of amylase enzyme was due to Kuhn (1925) and Ohlsson (1930). Kuhn showed that the sugars set free by the action of α -amylase, have the reducing group in the α -configuration (mutorotating downwards), while β -amylase liberated reducing groups (in maltose) in the β -configuration (mutorotating upwards). Ohlsson obtained both enzymes from malt diastase and further distinguished between them by demonstrating the random-fragmenting nature of the α -amylase in contrast to the endwise action of β -amylase on starch chains, whereby only maltose is liberated. Meyer (1952) has briefly summarized the early history of the amylases and the whole subject has been covered fully in a review by Whelan (1958).

In this work, β -amylase was employed to characterise amylose and amylopectin. This enzyme hydrolyses alternate linkages in amylose and in the external chains of amylopectin and glycogen with the

production of β -maltose. This necessitates a Walden inversion during the hydrolytic process. With the exception of phosphorylase, β -amylase provides the only method by which the anomaly present in amylose (Peat, Pirt and Whelan, 1952a) can be detected. This anomaly is however effectively removed by the action of Z-enzyme (Peat, Pirt and Whelan, 1952a). As the Z-enzyme is commonly found as a contaminant in β -amylase preparations (Whelan 1958), great care must be taken to ensure that the β -amylase used to characterise amylose is free from this impurity.

Preparation and purification of β -amylase:- The enzyme occurs abundantly in seeds and certain other parts of the higher plants. It has not been found in micro-organisms or in animals (Whelan 1958). Crystalline β -amylase has been obtained from sweet potato, barley, malt and wheat. Those materials also contained large amounts of α -amylase and it was only with great difficulty that enzymes free from Z-enzyme (weak α -amylase) were obtained. In this work the β -amylase used was prepared from soya-beans, by the procedure of Peat, Pirt and Whelan (1952b). Soya-beans have considerable advantages over other sources of β -amylase, in that they have present only very small quantities of α -amylase.

Ether-defatted soya-bean flour was shaken in water (5 ml/grm. of flour) for 2 hours. The suspension was then filtered through muslin before being centrifuged, the residual solid being rejected. The supernatant liquor was brought to pH. 4.8 by the addition of 1 N - sulphuric acid, the resulting precipitate being removed by centrifugation.

The solution was then heated to 60-61° C for 30 minutes, cooled in ice-water and the precipitate removed. Solid ammonium sulphate was added to give a 0.67% saturated solution and this precipitate formed was removed by centrifugation and dissolved in water. This solution was dialysed against running water for 48 hours. The resultant solution was then subjected to repeated fractional precipitation at pH. 3.6 using ammonium sulphate. Using this method the volume of solution was reduced to 10 ml.

Crude or stock soya-bean β-amylase was prepared according to the method of Bourne, Macey and Peat (1945). Soya-bean flour was shaken overnight with water, the solid removed by centrifugation and ethanol added to the supernatant to a final concentration of 60%. The precipitate was removed by filtration, washed with ethanol and dried over calcium chloride in vacuo.

Purity of the enzyme. - Preparations of β - amylase often have present impurities in the form of Z-enzyme or even maltase. As these enzymes will attack amylose and amylopectin they must be removed before the enzyme can be used. No efficient method is yet known for removing these enzymes without seriously impairing the activity of the β-amylase at the same time. Each preparation of β-amylase must therefore be tested to ensure the absence of Z-enzyme and maltase.

(a) Z-enzyme. - Digests containing amylose and the β-amylase in solutions buffered to pH. 3.6 and 4.6 were incubated for 24 hours at 35°C. It is known that Z-enzyme is inactivated at pH. 3.6, and therefore if the conversion to maltose was the same in each digest, then Z-enzyme

was absent from the sample. If however, there was at the end of the incubation period, amylose dextrin in the digest at pH. 3.6 and no dextrin in the 4.6 digest then Z-enzyme contamination was shown to be present.

(b) Maltase. - A digest containing maltose (ca. 0.5 mg/ml.) and enzyme was incubated at 35°C for 24 hours in a solution buffered to pH. 4.6. Any increase in reducing power, as detected by the alkaline ferricyanide-ceric sulphate method, signified the presence of maltase.

Activity of the β -amylase:- The unit of β -amylase activity used in the enzyme work was that defined by Hobson, Whelan and Peat (1950) i.e. the amount of enzyme which when incubated for 30 minutes at 35°C will liberate 1 mg. of maltose from a starch solution prepared as follows: starch solution (0.6%; 25 ml.); acetate buffer (pH. 4.6 4 ml.) and enzyme solution (1 ml.). The enzyme concentration was adjusted so that 10-20 mgs. of maltose were produced during the period of incubation.

The hydrolytic action of β -amylase. - There are only two possible mechanisms for the action of β -amylase on a polysaccharide substrate, these are - a single-chain attack or a multi-chain attack. Conflicting evidence as to the true action pattern of this attack on amylose, has been presented. Kerr (1949) indicated a single-chain action, but this was later opposed (Bourne and Whelan 1950) and a multi-chain action suggested. Kerr and Cleveland (1951) produced evidence of the single-chain action. This they obtained by a more detailed study of the reaction kinetics of the system. It was already known that β -amylase

attacked only the non-reducing end of the molecule. If the action pattern was multi-chain in nature then the number of non-reducing end groups would remain substantially constant during the reaction, which would therefore be governed by zero-order kinetics. If on the other hand a single-chain mechanism was preferred, the substrate concentration would be continually reduced during the reaction and first order kinetics would be obeyed. Kerr and Cleveland in 1951 obtained first order kinetics for the reaction system, which suggested that the mechanism of the enzymic degradation was of a single-chain type. In 1951 however this result was not universally accepted and Bird and Hopkins (1954) and Bailey and French (1957) produced evidence of multi-chain action and enzymic action intermediate between multi- and single-chain mechanism. Cowie et al. (1958) showed that amylose was degraded at pH. 4.6 and 35°C by a single-chain mechanism. Bryce et al. (1958) showed that the β -amylase attack of glycogen was of a multi-chain type. The obvious confliction of these results was clarified considerably by Banks (1960) who carried out a detailed study of the kinetics of β -amylase attack on amylose, amylopectin and glycogen. Banks results showed that whereas the β -amylase hydrolysis of amylose was of first order kinetics and thus a single-chain attack, that of amylopectin was a single-chain action for 70% of the enzyme attack and after this a multi-chain attack occurred. The results for glycogen showed that there was a very restricted phase of attack in which first order kinetics hold and on the whole a multi-chain reaction is prevalent.

Enzymic determination of amylose purity. - The concurrent action of

Z-enzyme and β-amylase completely hydrolyses amylose to maltose (Peat, Pirt and Whelan, 1952a). This thus presents a simple method whereby the purity of an amylose sample may be determined. If, in the presence of Z-enzyme, an amylose sample is not 100% converted into maltose, then the amylose is not pure and must contain contaminants. If, the impurity is taken as being amylopectin, then assuming that amylopectin has an average value of 55%, the percentage purity of the amylose can be easily determined. If however a value of 98-99% conversion into maltose is achieved, it is difficult on the evidence of reducing power alone, to decide if amylopectin is present or whether the remaining 1-2% is nearly due to experimental inaccuracy. In such a case, the addition of a few drops of M/100-iodine solution to a digest, can distinguish these alternatives. If no colour is produced then the departure from the expected 100% is due to experimental error, whereas a mauve stain indicates that contaminating amylopectin is present. Table 2.1. shows comparative purity results for a series of amylose samples and shows the good agreement between potentiometric titration results and enzymic assay.

Table 2.1.

	% Purity	
	Potentiometric titration	Enzymic assay
Amylose 1	98	100
Amylose 2	98	100
Amylose 3	94	96
Amylose 4	82	85

2d

Viscosity.

One of the most characteristic properties of solutions of high polymers is their high viscosity. Viscosity measurements are simple and accurate and have therefore been widely used for investigating polymer solutions.

Absolute viscosities are not often used in polymer work, the important quantities being relative.

Relative viscosity η_R = Ratio of viscosity of solution η to that of solvent η_o

$$\eta_R = \eta/\eta_o \qquad 2.01$$

Specific viscosity η_{sp} = Relative increase of viscosity

$$\eta_{sp} = \frac{(\eta - \eta_o)}{\eta_o} = \eta_R - 1 \qquad 2.02$$

η_{sp} is dependent upon the concentration and the ratio $\eta_{sp/c}$ is defined as the viscosity number. The limiting viscosity number or intrinsic viscosity $[\eta]$ is obtained by extrapolating the viscosity number to infinite dilution.

$$[\eta] = \lim_{c \to 0} \eta_{sp}/c \qquad 2.03$$

(In this thesis the concentration units are in grm/ml; as recommended by I.U.P.A.C. (J. Polymer Sci., 8, 257, 1952)).

The viscosity of a solution is dependent on the size and shape of the solute molecules. Molecules which have an extended shape will offer

a higher resistance to solvent flow than tightly curled molecules and hence their solutions will have higher viscosities for the same molecular weight.

Molecular Weight determination. The viscosity method is the simplest method used in molecular weight determinations involving dilute polymer sclutions. As the results obtained are however not absolute they must be correlated by other absolute methods. By visualising linear molecular as stiff rods Staudinger (1932) was able to relate the limiting viscosity number to the molecular weight by means of the linear function represented by. -

$$\eta_{sp/c} = K \times M \qquad \text{where } K = \text{constant}, \; M = \text{Molecular weight} \tag{2.04}$$

This equation was originally used for polymers of relatively low molecular weight. A more general equation relating the viscosity - molecular weight relationship for a large number of polymers in the molecular weight range 10^4 to 10^6 is -

$$[\eta] = KM^{\alpha} \tag{2.05}$$

where α is a constant and is a function of the geometry of the molecule in solution and has according to McGoury and Mark (1938), values between 0.5 and 2.0 for tightly curled and rigidly extended molecules respectively. K and α are both constants which can be found by carefully fractionating a polymer into samples of different molecular weights and determining the weight average molecular weight (M_w) for each sample. A graph of log $[\eta]$ VS log (M_w) then gives

a straight line of slope α and intercept K on the log $[\eta]$ axis. The constants must be determined for each polymer-solvent system.

The different methods for the measurement of a molecular weight of a macromolecule, give an average value, for there is present a mixture of polymer molecules of different sizes. Methods measuring such properties, as osmotic pressure, elevation of boiling point and chemical assay of the end groups are dependent on the number of molecules of each species present. Such methods give the number average molecular weight ($\bar{M}_n$). Other methods such as, light scattering and in certain cases, sedimentation measurements depend not only upon the concentration but also on the weight of each particle - these methods give a weight average molecular weight ($\bar{M}_w$).

For any system in which n_i is the number of molecules of species i and M_i the molecular weight of the species i,

$$\bar{M}_n = \frac{\sum Mi\ ni}{\sum ni} \qquad 2.06$$

$$\bar{M}_w = \frac{\sum Mi^2\ ni}{\sum Mi\ ni} \qquad 2.07$$

It is therefore obvious that M_w must be greater than M_n.

Viscosity measurements usually give an average molecular weight which is neither M_w nor M_n. Flory (1943) defined the viscosity average molecular weight $\bar{M}_v$ as

$$\bar{M}_v = \left[\frac{\sum Mi^{1+\alpha}\ ni}{\sum Mi\ ni} \right]^{\frac{1}{\alpha}} \qquad 2.08$$

where the α represents the exponent in the modified staudinger equation (equation 2.05). If $\alpha = 1$ then equation 2.08 reduces to the simple equation 2.07.

The effect of size and shape. - Viscosity measurements can give some idea of the approximate shape of a polymer molecule in solution. Considering the two extreme shapes, that of a rigidly extended, rod-like chain and that of a tightly curled chain, it is obvious that molecules of equal molecular weight will offer different resistances to solvent flow. The extended type of molecules will give higher viscosities than the curled molecules, because their shape will take up a larger volume in the solvent.

Simha (1940) deduced the equation -

$$\frac{\eta_{sp}}{c} = \frac{J^2}{15(\ln 2J - \frac{3}{2})} + \frac{J^2}{5(\ln 2J - \frac{1}{2})} + \frac{14}{15} \qquad 2.09$$

In this equation there is a relation between (J) the axial ratio i.e. length/diameter, and viscosity. The equation is, however, limited in its application, because whereas it holds for a rigidly extended molecule, it does not necessarily hold for a random coil which is the most probable shape for a plymer molecule in solution. When the solute molecule is large and Brownian movement predominates over velocity gradient, the limiting viscosity number may be related to the molecular weight by the equations of Huggins (1942) and Debye (1946). These are:

$$\text{for a rigid coil} = K_1 n^2 \qquad 2.10$$

$$\text{for a free draining coil} = K_2 n^1 \qquad 2.11$$

for an impenetrable sphere $= K_3 n^0$ 2.12

where n is the number of hydrodynamic units per chain and hence is directly proportional to the molecular weight. These have been modified to give equation 2.05, i.e.

$$[\eta] = K M^{\alpha}$$

where α varies from 0 - 2.

The above empirical equations may be superceded in the future by the theory of Flory and Fox (1950). As the limiting viscosity number has dimensions of volume per unit weight, it is considered to be proportional to the "effective volume" of the isolated macromolecule in solution devided by its molecular weight. This so called "obstructional volume" is proportional to the cube of a certain linear dimension of a randomly kinked molecule. Flory and Fox (1950) proposed that the cube of this critical length (i.e. the root mean square end-to-end distance) - $(\bar{R}^2)^{3/2}$ be split into two factors (a) $(\bar{R}_o^{\,2})^{3/2}$ which is the unperturbed value of $(\bar{R}^2)^{3/2}$ and (b) α^3 a volume expanding factor by which the linear dimensions of the molecular chains are increased owing to the intramolecular interactions i.e.

$$(\bar{R}^2)^{3/2} = (\bar{R}_o^{\,2})^{3/2} \times \alpha^3 \qquad 2.13$$

The Staudinger equation can therefore be modified to give

$$[\eta] = K M^{1/2} \alpha^3 \qquad 2.14$$

where

$$K = \Phi (\bar{R}_o^{\,2}/_M)^{3/2}$$

The volume expanding factor can be defined by the equation -

$$\alpha^5 - \alpha^3 = 2 C_m \gamma(1 - \Theta/T) M^{\frac{1}{2}} \qquad 2.15$$

where γ = entropy parameter; $(1 - \Theta/T)$ = heat of dilution term and C_m = constant.

Θ is that temperature at which α, in an inactive solvent, is unity, regardless of the molecular weight. At this point the molecular dimensions are unaffected by chain segment interaction. The parameter Φ is essentially independent of molecular weight and solvent and has a value of $(2.1 \pm 0.3) \times 10^{21}$. (R is expressed in cms. and $[\eta]$ in gm/100 mls.). Thus at the Θ point, $[\eta]$ determines the molecular weight according to

$$[\eta] = KM^{\frac{1}{2}} = (2.1 \times 10^{21})(\bar{R}_o^2/M)^{\frac{3}{2}} (M^{\frac{1}{2}}) \qquad 2.16$$

This theory requires a knowledge of $(\bar{R}^2)$ from light scattering data to supple ment viscosity measurements.

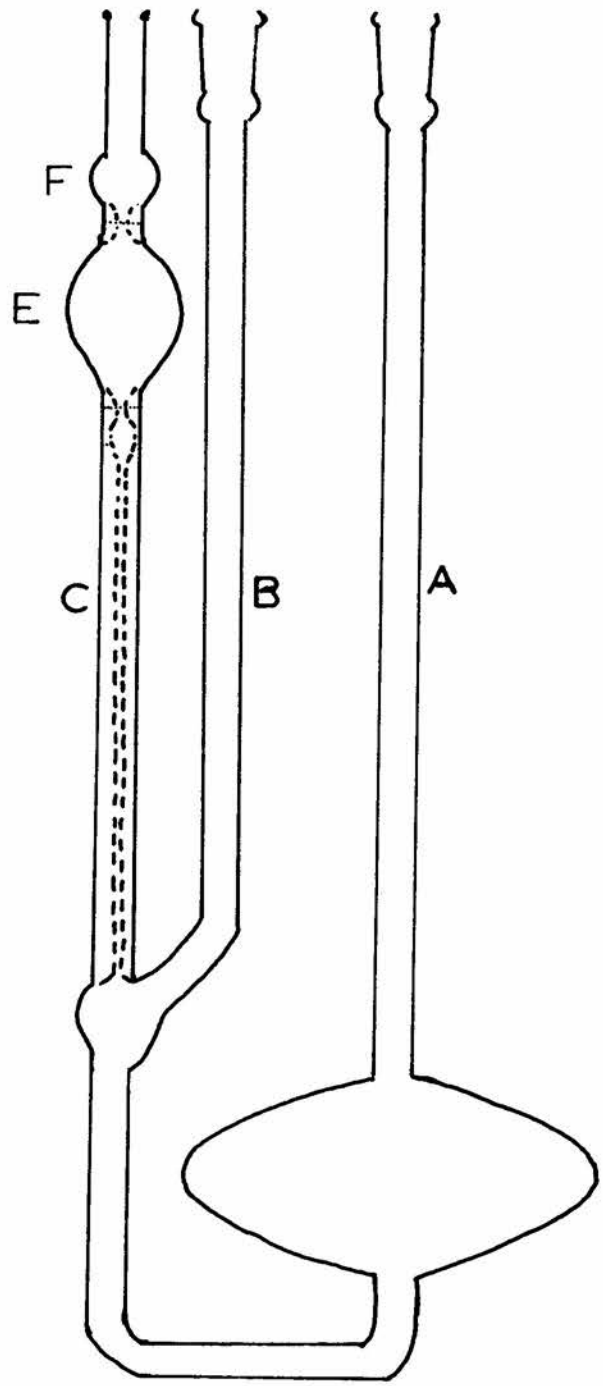
Apparatus and procedure

All viscosity measurements were carried out using a modified Ubbelohde visco meter (Ubbelohde 1937; Davis and Elliot 1949), with a minimum working-volume of 10 ml. (See Fig. 2.3). The viscometer was clamped firmly in a vertical position on a brass stand and determinations were carried out in a bath thermostated at 25 ± 0.01°C.

The filtered solution (10 ml.) was added by pipette down tube B and after allowing to come to temperature equilibrium, tube B was

FIG. 2·3

The Modified Ubbelohde Viscometer



closed with a glass stopper and pressure applied to A until the liquid level was at mid-point of bulb F. The pressure was then released, the stopper removed and the time required for the liquid level to pass two marks (one above and one below bulb E) was measured. These flow times were measured by stop watch to the nearest 0.1 sec.

Dilutions with filtered solvent (5 ml.) were made *in situ* by adding a given volume of solvent by a pipette down tube B, the solution mixed by gently blowing air down tube B, and the process repeated.

A more efficient procedure of viscosity measurement is that of pipetting initially 10 ml. of solvent into the visco meter and adding 5 ml. portions of the solution. This enables a quick check of the solvent flowtime and gives better spacing of the concentration. In both cases the initial solution was made up by weight or, if this was not possible, its concentration was determined by hydrolysis to glucose and estimation of the glucose by the alkaline ferricyanide/ ceric sulphate method previously described in 2a. In a capillary viscometer of this type, the viscosity is given by

$$\eta = Kdt - \frac{Bd}{t} \qquad 2.17$$

where, d is the density of the liquid, t is the time of flow and K and B are the viscometer constant and kinetic energy correction factor respectively. Using standard solvents, e.g. "AnalaR" acetone, benzene and butan-1-ol whose viscosities are known, the kinetic energy factor B can be calculated from the equation 2.17. The

value of B was found to be negligible for the Ubbelohde viscometer used. So

$$\eta = Kdt \quad \text{for the solution}$$

$$\text{and} \quad \eta_o = Kd_o t_o \quad " \quad " \quad \text{solvent}$$

The relative viscosity is therefore given by -

$$\eta_R = \frac{Kdt}{Kd_o t_o} \qquad 2.18$$

and the specific viscosity by

$$\eta_{sp} = \frac{dt - d_o t_o}{d_o t_o} \qquad 2.19$$

For dilute polymer solutions $d = d_o$

Therefore -

$$\eta_{sp} = \frac{t - t_o}{t_o} \qquad 2.20$$

Having thus obtained by experiment the values of η_{sp} for several concentrations, a graph of $\eta_{sp/C}$ against C will give a plot from which the $\lim_{C \to o} \eta_{sp/C}$ can be obtained. This is the limiting viscosity number or intrinsic viscosity $[\eta]$.

2e

ULTRACENTRIFUGATION

Introduction:- If the density of a material suspended or dissolved in a liquid, differs from that of the liquid, a force field applied to the system will separate the solute and solvent. The molecular size of the material being dissolved governs the force field required to cause separation. With materials of colloidal dimensions force fields many times greater than gravity are required to overcome diffusion and cause a sedimentation rate which is proportional to the molecular size. The most efficient method of achieving these high force fields is centrifugation at high speeds. The technical difficulties involved were overcome by Svedberg over 30 years ago, but it is only in the last decade that the development of the necessary instrument, has led to its widespread use as an analytical tool in polymer chemistry. The original work in this field was carried out by Svedberg and Pederson (1940) and the whole field of ultracentrifugation has recently been reviewed by Schachman (1959).

Ultracentrifugation therefore provides a relatively simple tool, whereby the molecular weight of a solute can be obtained. This can be achieved by (a) sedimentation velocity measurements in which the rate of movement of the solute is related to the molecular weight and (b) sedimenation equilibrium in which the applied centrifugal force is exactly balanced by the tendancy of the molecules to diffuse.

Theory of sedimentation velocity:- If a sufficiently large force is applied to a particle in solution, it will sediment in the direction of the applied field. Svedberg showed that the rate of movement of these molecules could be measured if certain conditions are fulfilled. One essential requirement is that no convection currents be set up, this he overcame by employing a sector shaped cell. After initial acceleration, the rate of sedimentation of the particle in the cell solution, will be constant, the centrifugal force being balanced by the frictional force. At a distance x from the axis of rotation the centrifugal force is $w^2 (1 - \bar{V}p)\, m$ i.e.

$$\text{Centrifugal force} = w^2 (1 - \bar{V}p)\, m$$

where w is the angular velocity, m is the mass of the sedimenting molecules and $\bar{V}$ is the partial specific volume. The frictional force is the product of the velocity $\frac{(dx)}{(dt)}$ and a frictional co-efficient f.

Therefore

$$w^2 (1 - \bar{V}p)\, m = f \frac{dx}{dt} \qquad 2.21$$

i.e.

$$\frac{dx}{dt} \quad \frac{1}{w^2 x} = \frac{m(1-\bar{V}p)}{f} \qquad 2.22$$

$$= S$$

The sedimentation co-efficient is the velocity of sedimentation per unit force field. But f, the frictional co-efficient can be related to the diffusion constant D; at infinite dilution

$$f_o = \frac{kT}{D_o} \quad \text{and thus by} \qquad 2.23$$

substitution in equation 1.17 and multiplying by N (Avogadros Number) we get

$$M = \frac{RTS_o}{(1 - \bar{V}p)\, D_o} \qquad 2.24$$

It is therefore possible to measure the molecular weight from sedimentation velocity data assuming that the partial specific volume and the diffusion constant are also known. The partial specific volume may be easily obtained by means of a pyknometer but the diffusion measurement is not so easily obtained and requires a specialised technique. It is therefore very convenient to eliminate the diffusion co-efficient and make use of the Scheraga-Mandelkern (1953) equation. By combination of viscosity and sedimentation measurements it can be shown that

$$\beta = \frac{NS_o [\eta]^{1/3} \eta_o}{M^{2/3} (1-\bar{V}p)} \qquad \text{where } \beta \text{ is a constant} \qquad 2.25$$

related to the axial ratio of the molecule.

Apparatus and Procedure - The instrument used was a Spinco Model E electrically driven ultracentrifuge (see Fig. 2.4), in which the drive motor (12,000 r.p.m.) is connected to the drive shaft through a direct step-up gear box (1:6) and this gives the machine a routine running speed of up to 60,000 r.p.m. the solution to be analysed was contained in a cell in which a sector shaped centre-piece was clamped between two quartz discs. These pieces were all firmly enclosed in a cylindrical cell housing. The cell was assembled and

FIG 2·4

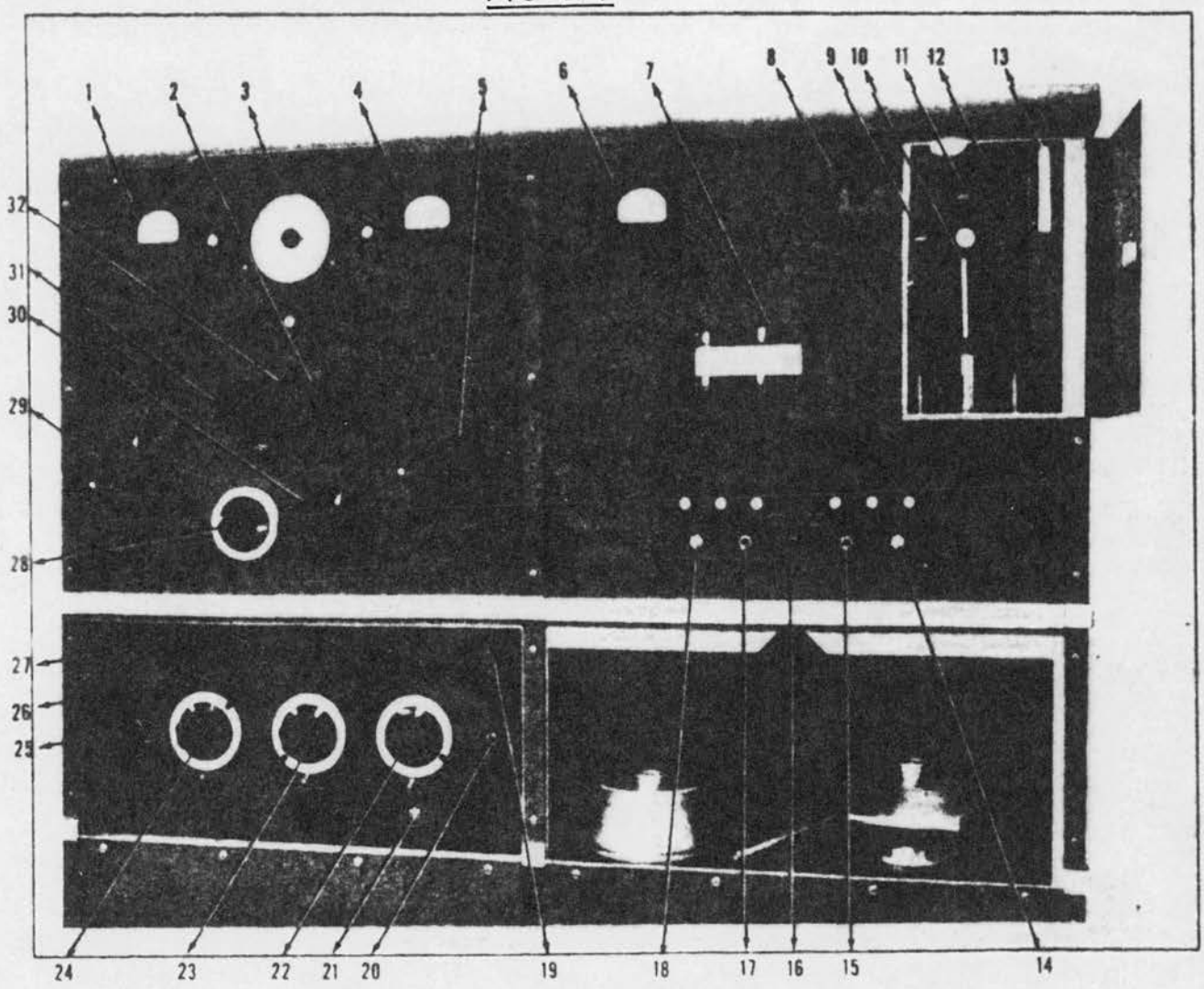


Fig. 2·4. INSTRUMENTS AND CONTROLS OF MODEL E ULTRACENTRIFUGE

1. Drive Motor Voltmeter
2. Individual Speed Selector Knob
3. Tachometer (Rotor Speed in 1,000th RPM)
4. Drive Motor Ammeter
5. Drive Motor Voltage Control Knob
6. Vacuum Indicator
7. Thermocouple Meter
8. Viewing Screen
9. Schlieren-Diaphragm-Angle Adjustment Knob
10. Reference Thermocouple Thermometer
11. Schlieren-Diaphragm-Angle Indicator
12. Photographic Plate Position Indicator
13. Slot Receiving Photographic Plateholder
14. Plate Shift Push Button
15. Plateholder Travel-Direction Switch
16. Thermocouple Selector Switch
17. Automatic Photo Switch
18. Viewing Screen Push Button
19. Vacuum Gauge Switch
20. Braking Rate Switch
21. Lightsource Intensity Switch
22. Lightsource Water Valve and Lightsource Switch
23. Diffusion Pump and Drive Motor Cooling Water Valve and Diffusion Pump Switch
24. Vacuum Chamber Air Valve and Vacuum Pump Switch
25. Vacuum Chamber Hoist Switch
26. Vacuum Gauge Adjustment Knob
27. Refrigeration Switch
28. Exposure Time Adjustment Dial
29. Time Switch
30. Exposure Interval Selector Knob
31. Speed Range Selector Knob
32. Speed Setting Indicator

the solution was injected into the centre-piece through a small hole by means of a needle and syringe. The cell was fitted into a hole (1" diam.), in the rotor (7½"), together with the cell counterpoise which balances the cell and which has in it two holes which act as reference marks. The Rotor containing analytical cell and counterpoise was connected to the drive, the vacuum chamber closed and the vacuum pumps started. Pressures of 1μ. and less were obtained by means of an oil diffusion pump backed by a rotary vacuum pump and runs were carried out under high vacuum to reduce thermal disturbances. A constant temperature of 20 ± 0.02°C was maintained by means of a Rotor Temperature Indicator and Control Unit. Sedimentation boundaries were detected by means of a Philpot-Svensson (1938, 1939) optical system. In this, light from a slit source is made parallel by a collimating lense and directed through the cell. In the region of the sedimenting boundary the light rays are deviated, due to the different refractive indices on either side of the boundary, after which they are converged by a condensing lense and reflected by a mirror on to an inclined bar as an image of the split source. The light then passes through a camera lense and a cylindrical lense before being reflected on to a viewing screen and photographic plate, as a schlieren pattern.

Calculation of Sedimentation Co-efficient:- Photographs of the sedimenting boundary were taken at various intervals and the distance from a reference line (5.73 cms. from the centre of rotation at

60,000 r.p.m.) to the mean of the peak was measured by means of a two-way travelling microscope. This was corrected for lens magnification M (2.215) and then converted to distance from the centre of rotation (x). Log X was plotted against t and S calculated from the Svedberg equation

$$S = \frac{2.303}{W^2} \times \frac{d\log x}{dt}$$ the angular velocity W 2.26

being measured during the run. Individual sedimentation co-efficients can be calculated from successive pictures from

$$S_1 = \frac{2\,(X_{t_2} - X_{t_1})}{(X_{t_2} + X_{t_1})\,W^2\,(t_2 - t_1)} \qquad 2.27$$

where X_{t_2} and X_{t_1} are the distances of the maximum ordinate of the sedimentation boundary from the centre of rotation at times t_2 and t_1 respectively. If the measurements are taken at equal time intervals then it is possible to use the formula

$$S_{AV} = \tfrac{1}{10}\,(2S_1 + 3S_2 + 3S_3 + 2S_4) \qquad 2.28$$

The average sedimentation co-efficient (S_{AV}) is equivalent to the value obtained by determining the slope of the line of least squares, of the log X versus t plot (Kegeles and Gutter, 1953).

Dependence of Sedimentation coefficient on concentration:- As molecular entanglement, solvation and hydrodynamic interaction are all affected by concentration, these effects have to be considered in sedimentation

coefficient experiments. This is especially true for materials of high molecular weight. At high concentrations the probability of entanglement is greatly increased, so that the molecules lose their individual character and there is no free sedimentation. As the concentration decreases however, the molecules begin to move more freely and consequently their velocity increases.

Many attempts have been made to deduce mathematical expressions which would fit concentration dependant data. Signer and Gross (1934) found that $1/S$ was a linear function of c for polystyrene in chloroform and Lauffer (1944) found that this also held for tobacco mosaic virus. Gralen 1944 proposed the equation

$$S = \frac{So}{1 + K_s C} \quad \text{where } K_s \text{ is a constant characterising} \qquad 2.29$$

concentration dependence. Newman, Loeb and Conrad (1953) found that $1/S$ against c did not give a straight line for nitrocellulose and proposed a plot of $(1/S - K_s c^2)$ against C. All these relations are however purely empirical and no mathematical equation has yet been deduced to describe the concentration dependence of all linear molecules. Amylose and amylopectin show marked concentration dependence. To eliminate this effect, sedimentation coefficients measured over a range of concentrations are extrapolated to zero concentration. This can be best achieved by plotting $1/S$ against c which gives a straight line. The So value can then be easily obtained from the graph.

2f

Diffusion.

Introduction. -

Equation 2.24 (the Svedberg equation) can be used to calculate the molecular weight of a polymer.

$$M = \frac{RTS_o}{(1-\bar{V}p)\, D_o}$$

S_o can be obtained from sedimentation results on different concentrations of the polymer, and the partial specific volume is also easily obtained by use of the pyknometer. The diffusion constant D_o can however only be obtained using specialised techniques.

Theory of diffusion. - Consider a system in which a solution, concentration C_o, is in contact with its solvent. If it is assumed that there are no external forces acting on the solute molecules, other than the osmotic pressure and gravity, the rate of diffusion is given by Ficks Law which states

$$\frac{dm}{dt} = -DA \frac{dc}{dx} \qquad 2.30$$

where dm is the quantity of solute which in time dt diffuses across a boundary of cross section A under the influence of a concentration gradient dc/dx and where D is the diffusion coefficient in cm^2/sec.

The diffusion can be followed by measuring the relation between C and X at various time intervals t. This can be done by measuring changes in refractive index n as $C \propto n$. The diffusion constant can then be obtained by the Wieners (1893) equation -

$$\frac{dn}{dx} = \frac{n_1 - n_o}{2\sqrt{\pi DT}} e^{-\frac{x^2}{4DT}} \qquad 2.31$$

where D is the diffusion constant, t the time, x the distance from the original boundary and n_o and n_1 the refractive indices of solvent and solution respectively.

The system may be represented by Fig. 2.5. The boundary in the diffusion cell is at the point x = 0. If the system is scanned by means of an interferometric optical system, then the curves in Fig. 2.5 are obtained. These may be changed into $\frac{dc}{dx}$ v. X curves by arithmetical differentiation. If Schlieren optics are employed then the differential curves are obtained directly. In the ideal case, these curves are the shape of Gaussian distribution curves and are equal with respect to area.

There are various methods for evaluating D.

(1) Maximum ordinate or Area method

At X = 0 on Fig. 2.5, the exponential term in equation 2.31 is unity and $(\frac{dn}{dx})_{x=0} = Hm$ where Hm is the maximum ordinate.

thus

$$(\frac{dn}{dx})_{x=0} = H_m = \frac{n_1 - n_o}{2\sqrt{\pi DT}} \qquad 2.32$$

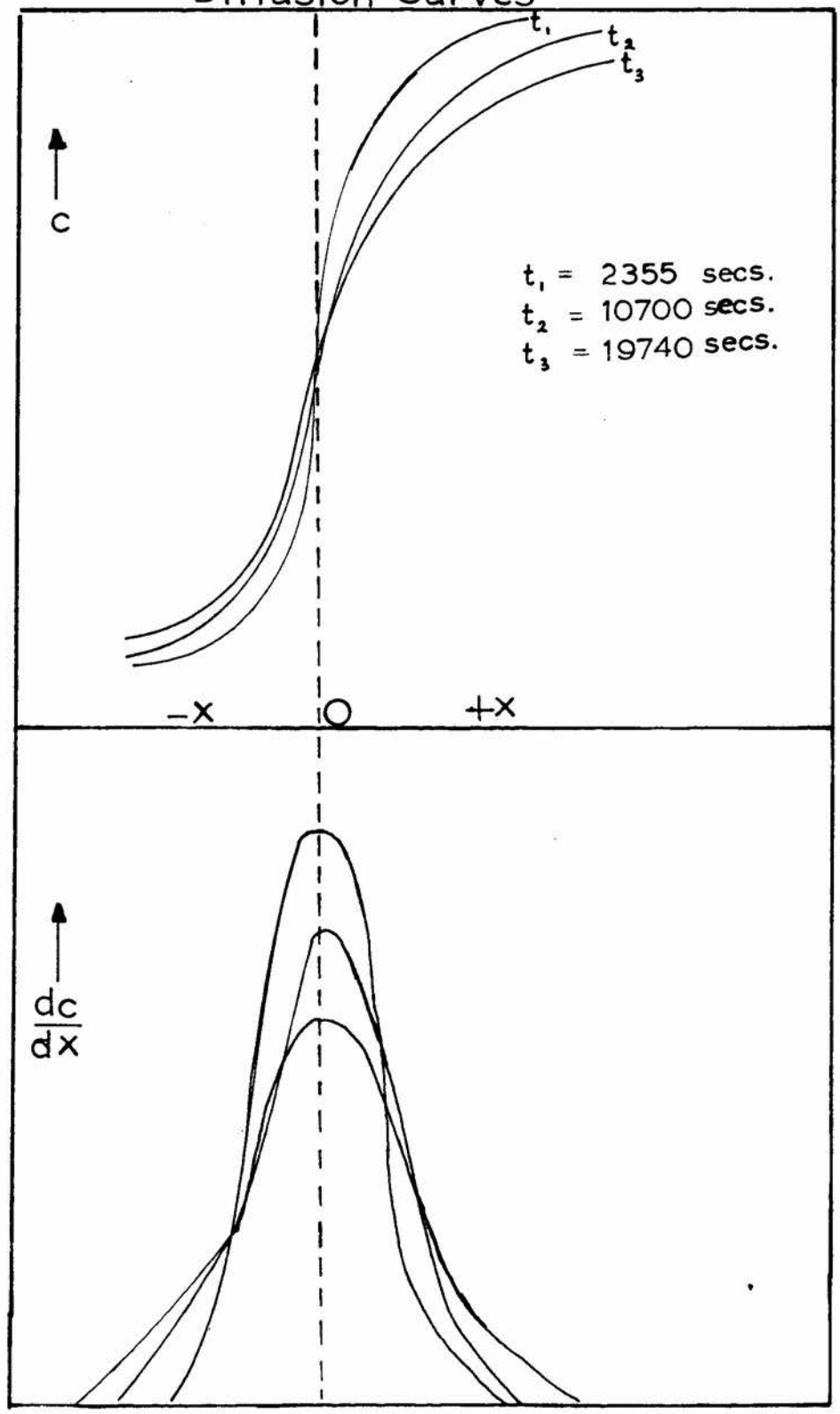
and therefore

$$D = \frac{(n_1 - n_o)^2}{4\pi t H_m^2} \qquad 2.33$$

Now the area (A) between the curve and the x - axis is given by

$$A = \int_{n_o}^{n_1} \partial n/\partial x \; x \; dx = n_1 - n_o \qquad 2.34$$

FIG. 2·5
Diffusion Curves



So

$$D = \frac{A^2}{4\pi t\,(H_m)^2} \qquad 2.35$$

This method of obtaining D is known as the maximum ordinate method or area method. Here D is obtained from a graph of H_m against $1/\sqrt{t}$ the slope of which is $\frac{A}{2\sqrt{\pi D}}$. The area A is obtained by means of a planimeter or by graphical integration.

(ii) Moment method

In this method, equation 2.31 is modified to give. -

$$dn/dx = \frac{n_1 - n_o}{\sigma\sqrt{2\pi}}\; e^{-x^2/2\sigma^2} \qquad 2.36$$

where $\sigma^2 = 2DT$ and σ is the standard deviation which is related to the second moment of the curve ($\mu_2{}^o$) about the centroidal ordinate by

$$\sigma = \sqrt{\mu_2{}^o} \qquad 2.37$$

Here the centroid ordinate is the one about which the first moment is zero. In practice the curve is divided into sections of equal breadth numbering outwards (S_1, S_2, ...S_n) from an arbitrary origin near the centre of the base line. If S_i is the vertical height of the strip numbered s_i then the first and second moments are given by

$$\mu_1' = \frac{\sum(si\ Si)}{\sum(si)} \quad \text{and} \quad \mu_2' = \frac{\sum(si^2\ Si)}{\sum(si)} \qquad 2.38$$

and the position of the true centroid ordinate is given by

$$X_o = S_o - \mu_1' \qquad 2.39$$

The true second moment is then

$$\mu_2 = \mu_1' - (\mu_1)^2 \qquad 2.40$$

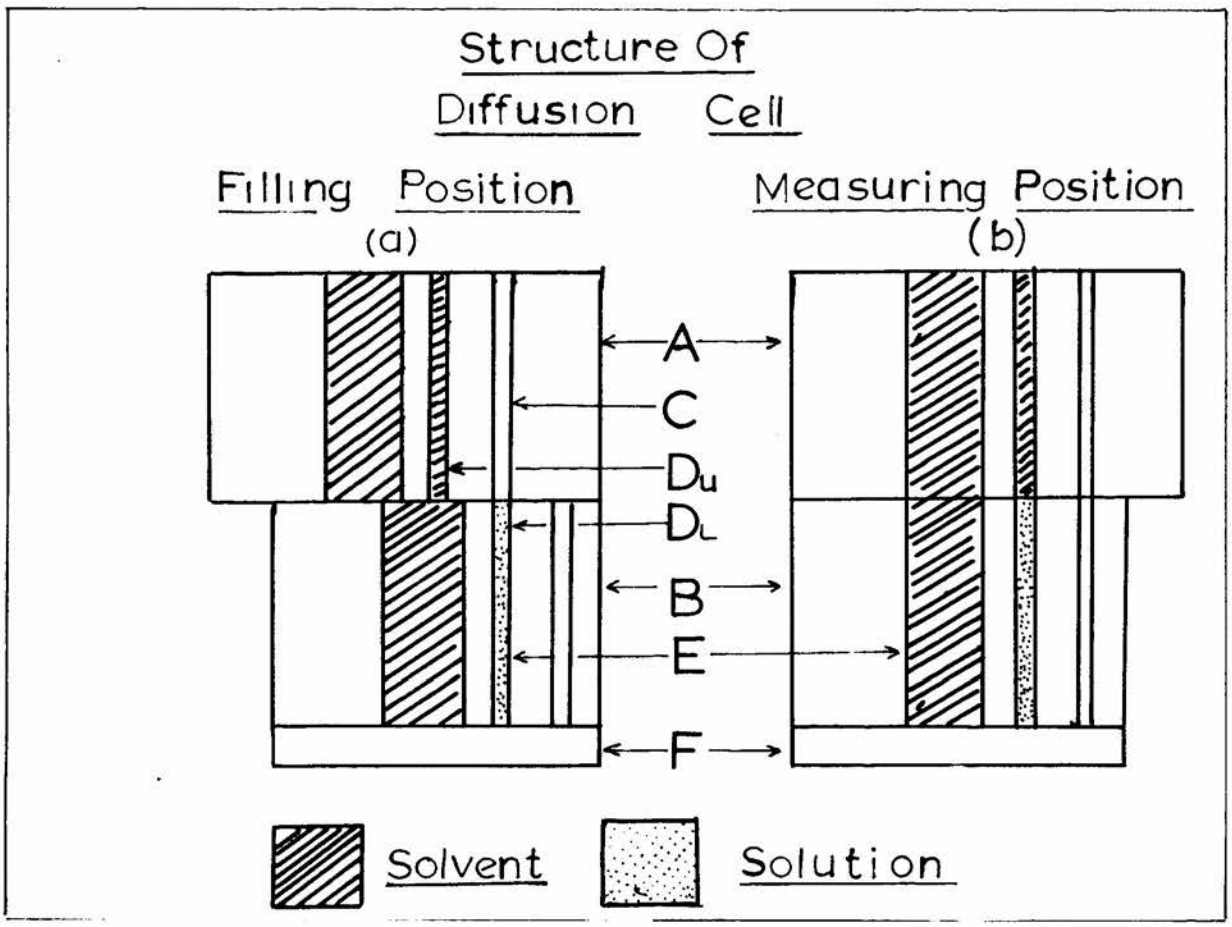
This second moment is however dependant on the width of the sections used and has to be converted into absolute units by. -

$$\mu_2^{\circ} = \mu_2 \omega^2 \qquad 2.41$$

The value of D is now obtained by graphing σ^2 against t. (Colloid Science Vol. 1. p. 246).

Apparatus and procedure. - Diffusion measurements were carried out on an Antweiler micro-electrophoresis and diffusion apparatus at 20°C. This is shown in Figure 2.6 and consists of two quartz blocks A and B, an upper and lower block. Block A which was coated on the underside with a thin film of vaseline was placed on block B in the filling position (Figure 2.6). The compartments of the blocks were then closed by placing on plate F. The solution of the materials whose diffusion constant was to be measured, was placed in D_L by means of a syringe, until the level reached the lower part of C. The compartment E which is the comparison compartment and the top half of the measuring compartment Du were then filled with solvent. The positioning of the top plate now completed the apparatus. The total cell system was placed in a cell holder attached to the Antweiler apparatus and allowed to come to temperature equilibrium

FIG. 2.6


Structure Of
Diffusion Cell
Filling Position
(a)
Measuring Position
(b)
A
C
Du
DL
B
E
F
Solvent
Solution

(2 hours). A mechanical screwing device enables block B to be moved under block A, after the temperature equilibrium has been reached. Great care must be taken with the alignment, to ensure that no secondary effects such as mixing occur which will completely invalidate the experiment.

After sufficient time had elapsed for a measurable concentration gradient to form, readings were taken using a Jamin interferometer. These readings were taken thereafter at regular intervals. The method of measuring using the Jamin interferometer requires the simple reading of interference fringes. In the interferometer a light ray is split into two by a Jamin prism. One ray passes through the comparison channel and the other through the measuring channel. A second prism combined the two rays and the alternate reinforcement and cancellation of the wave-fronts gave interference fringes. Now if the optical path length of the two beams is different due to the different refractive indeces of solvent and solution then there will be a displacement of the fringes. In measuring the concentration gradient, one of the fringes was chosen as the zero line and the fringe displacement measured by returning this reference fringe to its zero position and then taking the readings from a micrometer drum. Values were taken every 0.2 mm through the gradient curve and a final plot of refractive index (n) against distance travelled (x) was then made.

An alternative photographic method could be employed to obtain the differential curves directly by means of a Schlieren optical system and a 35 mm camera.

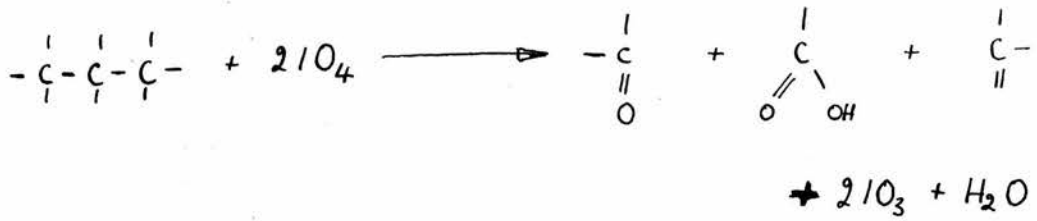
2g.

Periodate Oxidation.

For many years periodate oxidation has been one of the basic methods used to elucidate the structures of many polysaccharides. Chain-length studies on amylose and amylopectin were originally carried out using the methylation technique of Haworth, followed by separation of the methylated sugars obtained on acid hydrolysis (see Hawerth and Machemer 1932). The chain length could then be easily calculated from the amount of 2:3:4:6-tetra- σ-methylglucose which had originated from the non-reducing end-groups. The accuracy of this method was increased by the introduction of chromatographic techniques for separation of the sugars formed by the methylation process.

This end-group assay of starches or their components can be replaced by the simpler decigram-scale methods of periodate oxidation. The selective oxidative fission of α-glycols by the periodate ion, giving two aldehyde groups was discovered originally by Malaprade (1928). This reaction was first applied to sugar ring-forms by Jackson and Hudson (1936) who later applied this reaction to starch and cellulose.

For 1,2,3-triols the oxidation by periodate, results in the production of one molecule of formic acid and the formation of two aldehyde groupings, two molecules of periodate being reduced in the reaction.



This reaction is valid for glycol groups in cyclic structures. Thus a chain of twenty α -1:4-linked D-glucose units, in which the reducing end-group forms a 1:6 linkage to another similar chain as in an amylopectin-type molecule, will consume twenty-one molecules of periodate i.e. 1.05 molecules per anhydroglucose unit, since the non reducing end-group alone consumes two molecules liberating one molecule of formic acid.

If this technique is used under accurate conditions, with due account taken of reactant blanks and accurate titration of the released formic acid, the average length of the unit-chain can be calculated. Originally, Halsall, Hirst and Jones (1947), used sparingly soluble potassium metaperiodate at 15°C for 2-10 days, as the oxidant but a modification of this technique using sodium metaperiodate at 2°C for 25 hours has since been devised by Potter and Hassid (1948).

The simplicity of application of the periodate reaction has contributed to its wide usage. The aqueous solvent conditions called for in the oxidation lend themselves to use with water-soluble carbohydrates. The quantitative nature of the reaction, together with the precise analytical procedures available for following its

course, make it possible to obtain a great deal of information, from experiments utilizing minimal quantities of materials. "Selective" oxidation by sodium metaperiodate, which is required if the maximum amount of knowledge regarding the structure of the carbohydrate is to be obtained, is governed by precise experimental conditions. Many different methods have been employed in the periodate oxidation of polysaccharides with the result that a considerable variety of values for unit-chain lengths have been obtained. In the ideal reaction there must be complete periodate oxidation i.e. selective oxidation without over-oxidation occurring. Polysaccharides of the α-1:4-glucosan type are easily over-oxidised. To minimise this tendancy, Hirst et al (1947) used the sparingly soluble potassium meta-periodate at room temperature, the calculated excess of oxidant and of formic acid released being kept low. Theoretically oxidation was completed in about a week. Potter and Hassid (1948) introduced the use of a saline solution of sodium metaperiodate at 2°C for 25 hours, but these conditions were thought to result in incomplete oxidation (Manners 1953).

A considerable variation of chain-length results for amylopectin have resulted because the formic acid released in the oxidation had been titrated with standard alkali to different end-points. For example titration with alkali to the following end-points have been used:- methyl red (Hirst et al, 1947); phenol phthalein (Hughes and Nevell, 1948); phenol red (Jeanes and Wilham, 1950) and mixed

methyl red/methylene blue (Schlamowitz 1951). · Others have titrated the formic acid potentiometrically. Of these Kerr and Cleveland (1952) titrated to pH. 7.1 while others titrating to pH. 5.5, 6.0, 8.0 and 8.2 have been referred to by Morrison, Kuyper and Orten (1953). Different methods of titrating residual periodate e.g. Fleury and Langes (1933) method or arsenite titration methods gave different results. Before a study of unit chain-lengths of α-1:4-glucans such as amylopectins and glycogens can be made the optimum conditions for an ideal reaction must be determined. This involves consideration of (a) the oxidant, (b) the solvent, (c) light effects, (d) temperature, (e) concentration of reactants and finally (f) methods of analysis with regard to titration blanks, end-points etc.

Optimum conditions of Periodate oxidation. - (a) The oxidant. The oxidant used in all the oxidation experiments was sodium metaperiodate (0.58 M).

(b) The solvent. The use of water or of an aqueous solvent in the reaction mixture is a characteristic of periodate oxidations as performed in the field of polysaccharide chemistry. Potter and Hassid (1948) and Halsall et al (1947) both used 1.5% sodium chloride solution as solvent. This gave complex and erroneous correction blanks and distilled wate r was found to be the most suitable and efficient solvent.

(c) Light effects. The effect of light on periodate oxidations has been studied by various workers. Head and Hughes (1952) have found that the formaldehyde and other secondary compounds produced in

periodate oxidations of polysaccharides are further oxidised in the presence of periodic acid and light but that no oxidation takes place in the absence of light. All sodium periodate reactions are therefore carried out in darkness.

(d) Temperature. The oxidation temperature for all experiments was 2°C. At this temperature, the periodate oxidation was complete in 25 hours.

(e) Concentration of the reactants. Various workers have used different concentrations of sodium metaperiodate in their oxidations. After considerable study of the oxidation, using different concentrations of the reactants, had been carried out, the following method was found to give the most reproducible results. About 200 mg. of the amylopectin or glycogen were dissolved up in 80 ml. distilled water and cooled to 2°C. Sodium metaperiodate (20 ml, 0.58 M) was then added and the mixture after shaking was left at 2°C. Ten ml. samples were removed at regular intervals for analysis, at intervals over a period of 48 hours. The excess periodate was decomposed by adding 0.5 ml. ethylene glycol, and the sample was shaken in the dark at room temperature for 30 minutes. The formic acid released in the oxidation was titrated with 0.01 M sodium hydroxide (semi-micro burette) in a nitrogen atmosphere.

(f) Analysis of results. Two basic methods which have been used to analyse the results of periodate oxidation are (1) periodate uptake and (2) formic acid release. The most successful method - and the simplest - is the titration of liberated formic acid with dilute

caustic soda after the addition of ethylene glycol (as above). The ethylene glycol added and shaken with the reaction mixture before titration ensures that there will be no excess oxidant in the solution which might continue to act during the titration.

Although periodate-oxidation is a much-used tool in organic chemistry, the oxidation of polysaccharides requires very fixed and accurate conditions of oxidation and assay if reproducible results are to be obtained. For example the ethylene glycol used to kill the excess periodate should be distilled free from acid contaminants, and the dilute alkali used in the titration should be accurately standardised against potassium diphthalate. The most effective cause of the variations of results in periodate - oxidation is due to the different pH. values taken by different workers to be the end-point of the formic acid - dilute alkali titration. A critical study was made of this titration and as can be seen from the graphs (Figure 2.7), titration figures pointed to an end-point of 6.25 (cf. Anderson and Greenwood 1955). This was carefully checked using the method of Gran (1952) to mathematically calculate the end-point. This modified Sorensen method gave the same end-point for the titration as the potentiometric method.

Another basic reason for the variation of results using this method of analysis is the nature of the correction blank. Figure 2.8 shows that different blank solutions gave different amounts of acid material which will be titrated along with the formic acid liberated in the oxidation. The Potter and Hassid (1948) conditions

FIG. 2·7

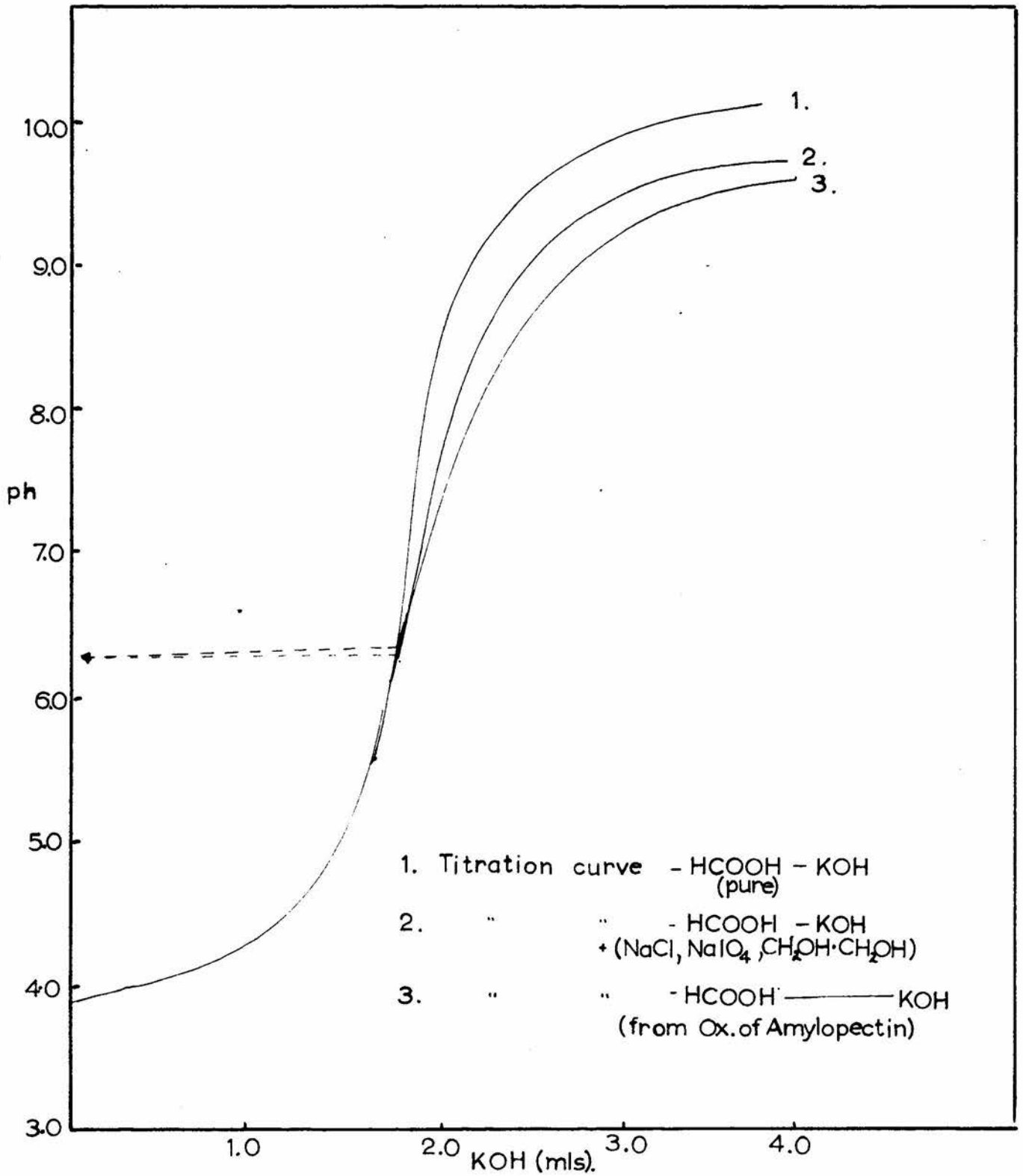
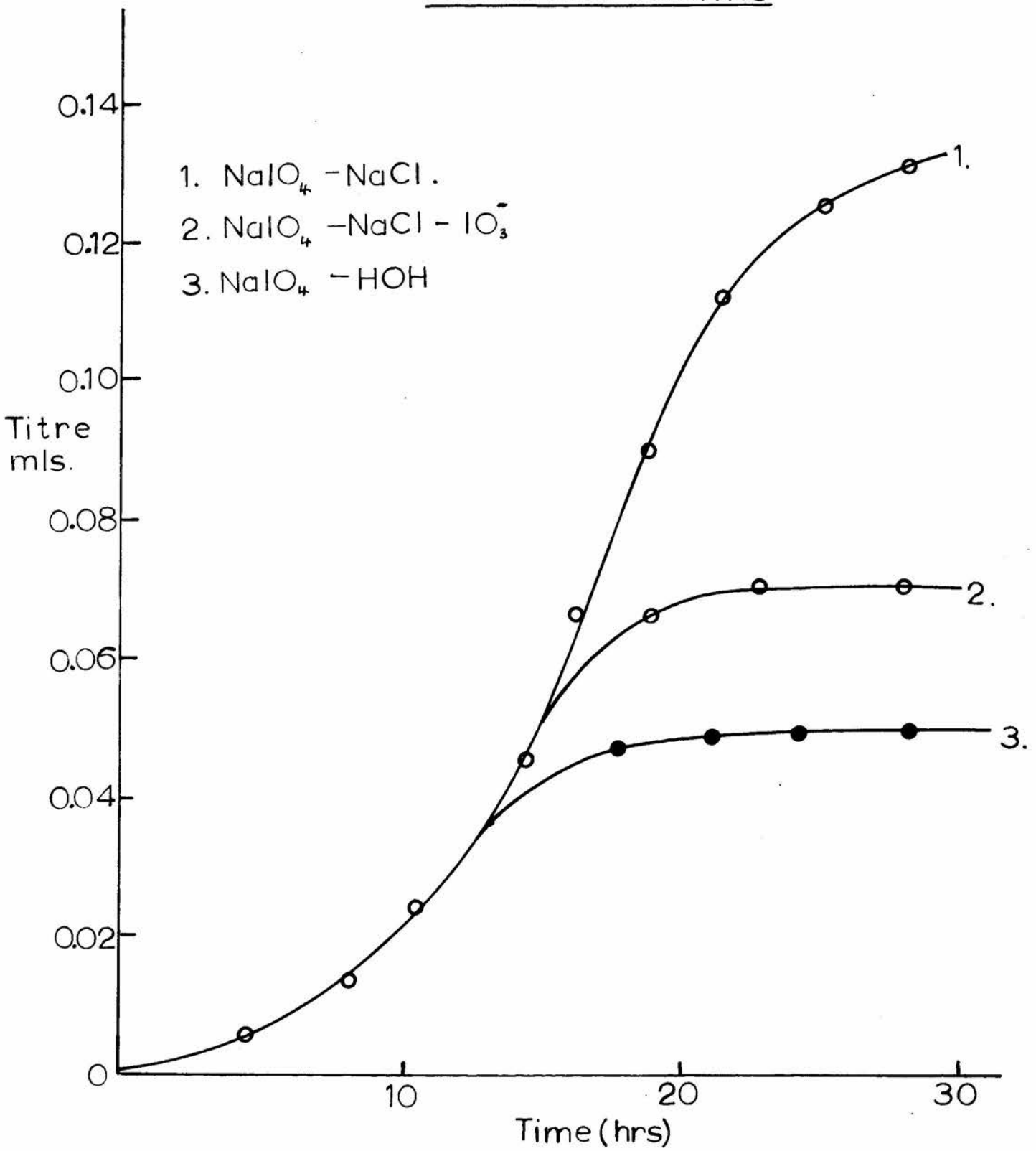

10.0
9.0
8.0
ph
7.0
6.0
5.0
4.0
3.0
1.0
2.0
3.0
4.0
KOH (mls).
1.
2.
3.
1. Titration curve - HCOOH – KOH (pure)
2. " " - HCOOH – KOH + (NaCl, NaIO4, CH2OH·CH2OH)
3. " " - HCOOH ——— KOH (from Ox. of Amylopectin)

FIG. 2·8

Titration Blanks



of oxidation using 1.5% NaCl solution as solvent gave a very large blank and was the cause of the discrepancies in chain-length results determined by this method. When the solvent was water the blank was constant after 20 hours and quite small. It was also interesting to note that the iodate produced in the oxidation stabilised the blank.

If care is taken in the method of periodate-oxidation reproducible and accurate results can be obtained.

2h

Estimation of Phosphorus.

Many starches contain phosphorus. The amount present varies considerably depending on the botanical source of the starch. Most starches contain only a little phosphorus (0.005-0.015%), although potato starch has been reported with as much as 0.1%. After starch fractionation the phosphorus was found to remain predominantly with the amylopectin fraction and little or none with the amylose fraction. Why this should be the case, and its ultimate effect on the properties of the starch components is not fully understood, although there is some evidence that the β-amylolysis limit of the starch fractions may be effected by the phosphorus present in the molecular structure acting as a barrier to the enzyme

Analytical Method.

Reagents - Ammonium molybdate-sulphuric acid solution. Ammonium molybdate (10 g) was dissolved in distilled water (100 ml) To this was added a solution of sulphuric acid (sp. grav. 1.84, 10 mls) and distilled water (100 ml).

Determination of the phosphate content of amylopectin - The amylopectin must be totally destroyed to enable the liberated phosphate group to be estimated. This has been carried out by several different methods. Phifer (1957) used the modified Moosmüller process in which the amylopectin was destroyed by sulphuric acid and hydrogen

peroxide. The process used in this work was that of Gieseking, Snider and Gatz (1935), and Smith (1953). This was the wet-oxidation process in which the amylopectin is destroyed with conc. nitric acid and perchloric acid. In this reaction 100 mg of dried amylopectin, contained in a 25 ml conical flask was mixed with approximately 0.25 ml concentrated nitric acid and heated on a hot plate. After the initial reaction had subsided, the mixture was boiled until almost dry. After removing from the hot plate, 0.5 ml of a mixture of equal parts nitric (8N) and perchloric acids (70%) was added. The solution was then heated gently until all the organic material was destroyed as indicated by a colourless solution. After evaporation, the residual salts were dissolved in 0.5 ml. hydrochloric acid (2N) and this solution used for the determination of phosphate, by the Fogg and Wilkinson (1958) method. The acid solution obtained after the destruction of the organic matter was diluted to ca. 40 ml., and 4.0 ml of ammonium molybdate-sulphuric acid solution added. Ascorbic acid (ca. 100 mg) was now added and the solution boiled for 1 minute, a blue colour being produced. After cooling, the solution was diluted to 50 ml in a graduated flask and the absorbance measured by means of a ultra-violet absorbiometer at 675mμ. A blank control determination in which the polysaccharide was omitted was carried out to determine the phosphate content of the reagents. A standard graph of ultra-violet absorbance against phosphorus content for aliquots of a standard solution of KH_2PO_4 enabled the phosphorus content of the polysaccharide to be determined.

Section 3

The Fractionation Of Starch And Its Components

3a.

Fractionation

Introduction

The heterogeneity of starch was recognised in the early literature by such investigators as Guerin-Varry (1834-36), Naegeli (1862-81) and A. Meyer (1881-95) but lack of systematic nomenclature tended to retard progress in this field. Much of the early confusion in the literature was systematized after 1942 when Schoch carried out successful quantitative separation of the two components. (See review by Schoch (1945)). Many methods of starch fractionation were developed by Schoch, but as few physicochemical methods for characterising the starch components existed in the 1940's slow progress was made.

The methods developed by Schoch and previous workers depend mainly on the differing properties of the two polysaccharide components of starch, in aqueous medium and can be classified in four general methods.

Various methods have been available for fractionating starch as follows:

1. Aqueous leaching of gelatinised starch. - This method involved extraction of the swollen, but intact granules with successive portions of hot water. Separation is effected by sedimentation, filtration or centrifugation. The soluble extract represents the crude (amylose) and the residue of swollen granules the crude (amylopectin).

2. Electrophoresis. - This method was developed by Samec (1935), and utilizes the fact that the phosphate groups associated with the amylopectin cause it to migrate towards the anode leaving the amylose in the supernatant. This gives a slow, incomplete fractionation and is accompanied by considerable retrogradation.

3. Selective retrogradation. - When a starch sol is allowed to stand the amylose slowly retrogrades and precipitates from solution. This is an extremely unsatisfactory system as co-precipitation occurs and only superficial fractionation is obtained (Maquenne and Roux, 1905).

4. Selective precipitation. - This method was developed by Schoch (1942) and affords the most successful method of fractionating starch into its two components, which are neither chemically degraded nor physically impaired.

The development in recent years of new physicochemical methods for the characterization of the starch components and the study of the size and shape of the macromolecules, has facilitated systematic studies on (1) the fractionation of starch and (2) the separate components. But for fundamental studies, the starch itself and its separate components must be isolated by a method that ensures minimum degradation and maximum purity.

Isolation And Purification Of Starches

In this work starch has been isolated and purified from a variety of botanical sources. The latter can be subdivided into four distinct groups.

Seed Starches	Fruit Starches	Root Rhizome and Tuber Starches	Seaweed Starches
Barley	Banana	Potato	Floridean
Malted Barley	Apple	Iris (Germanica)	
Broad Bean	Mango	Parsnip	
Rubber Seed			
Smooth Pea			
Wrinkled Pea			
Amylomaize			

With the exception of Floridean Starch, where a specialised technique was required (see section 4), all the starches were purified in the same way, by purely physical methods, which should give maximum yields of starch with the minimum amount of degradation.

Experimental.

The plant material was in the first instance, kept in 0.01 M-mercuric chloride solution to inhibit enzymic action, and extracted in an "Atomix" blender for 2 minutes. The pulp, so obtained was filtered through muslin and the filtrate immediately centrifuged. After discarding the supernatant, the starch was washed repeatedly by sedimentation in 0.1M - sodium chloride solution. After the residual pulp had been re-extracted three times, the crude starch products were combined, suspended in saline solution and shaken with toluene (1/10 vol.) for 12 hours to denature the protein (Greenwood and Robertson 1954). The starch was then allowed to settle and the toluene layer

containing coagulated protein discarded. This process was repeated several times until all the protein had been removed (tested by micro-kjeldahl) and the starch was pure. The starch was then defatted by treating with boiling 80% aqueous methanol and the purified defatted starch stored under methanol.

It is very difficult in many cases to get a pure starch. Cereal starches, for example, contain considerable amounts of proteinous material, and many toluene-extractions are required to purify them. Fruit starches are very often contaminated with fibrous material, which is so small that it cannot easily be separated from the starch. In the Banana, Apple and Mango starches, reported in this work, noeffort was made to remove the fibrous material until the starch had been dispersed, when the fibrous material appeared as an easily removable layer in the Sharples super-centrifuge.

3b.

Characterisation of Starch Granules

(a) Microscopic examination. - All the starches were microscopically examined in aqueous suspension. A polarised microscope fitted with Ahrens prisms and capable of magnifications of up to 500 diameters was used. Initial inspection in ordinary light yielded considerable information as to the state of the granule with regard to size, extent of compound granule formation, enzymic corrosion etc. (see review by Badenhuizen 1959), whilst examination using polarised light enables their birefringent properties to be studied. By photographing the granules and enlarging the photomicrographs, the size distribution of the starch granules was determined.

(b) Gelatinisation temperature. - A Kofler electrically-heated microscope stage was used to determine the gelatinisation temperature i.e. the temperature at which the starch granules lose their birefringent properties when heated in a swelling medium. However in a given starch sample the granules do not gelatinise simultaneously, a gelatinisation range of 5-10°C is usually observed. Hence it is necessary to quote the temperature of initiation and termination of the gelatinisation process (Schoch and Maywald 1956). In order to determine the gelatinisation range, a drop of starch suspension (ca. 0.1%) was placed on a microscope slide and surrounded by a ring of viscous mineral oil. A cover glass was then placed carefully in position so that no air bubbles were formed and the oil seal remained unbroken. The slide was placed on the hot stage, which was then covered by a glass

plate to ensure uniformity of temperature during heating. The setting on the variable transformer was adjusted so that when approaching (and also during) the gelatinisation range, the rate of temperature rise did not exceed 2°C per minute. Continuous observations were made under normal lighting, but when the granule started to swell the Ahrens analyser was introduced to show the loss of birefringent properties. By measuring the temperature at which various percentages of granules (initiation, 10, 25, 50, 75%, completion) lost their birefringent properties gelatinisation curves were obtained.

(c) <u>% Protein.</u> - As the starch granules mature, they increase in size and fill the amyloplasts. Many of these granules are surrounded by a protein-layer which makes it very difficult to purify the granular starch. The amount of protein present is easily obtained by determining the nitrogen content of the starch by the semi-micro-kjeldahl method. % Protein = % N_2 x 6.25.

(d) <u>Iodine affinity.</u> - This procedure is explained in section 2b. It is based on the potentiometric titration method of Bates, French and Rundle (1943). The percentage amylose present in starch varies considerably. Waxy starches may have as little as 2% amylose present while wrinkled pea (66%) and amylomaize (52%) have very high amylose content. The amylose content is of extreme commercial importance and in the dextrin industry a starch of high amylose content, where the amylose is easily accessible, is in great demand.

3c

The Fractionation of Starch

The most successful method of fractionating starch into pure undegraded amylose and amylopectin, is the method developed by Schoch and his co-workers (1942). By this selective precipitation method, the linear fraction is selectively precipitated by a polar organic compound such as pentanol, cyclohexanol or thymol. This method ensures that the fractions obtained are neither chemically degraded nor physically impaired. The efficiency of a starch fractionation depends to a considerable extent on the structure of the granule. The more intense the "micellar" structure of the granule the more difficult it is to fractionate the starch. This variation in "micellar" structure is reflected on the gelatinisation temperature of the starches. Potato starch granules have a gelatinisation temperature range of 61-64° and are easily dispersed in boiling water. Wrinkled pea starch, on the other hand, although having a very high amylose content (66%), has a compound "micellar" structure, a high gelatinisation temperature and is very difficult to disperse and therefore to fractionate. It is essential that the granules of such a starch should be pretreated to render them more amenable to fractionation.

The pretreatment of starches. - Several methods have been suggested, but great care must be taken to ensure that the method

used does not cause inadvertant degradation, to either of the starch components. Of the methods suggested (a) boiling organic liquids (Montgomery and Senti, 1958), (b) 1-$\bar{M}$-caustic alkali at 0°C (Potter et. al., 1953) and (c) liquid ammonia (Hodge et. al., 1948) have been most successful. The most efficient method, least liable to give degradation - was found to be method (c). In this method an alcohol-suspension of starch (10-15 grm)was treated for 15 minutes in a Dewar vessel with liquid ammonia (100 ml.). After this time, the mixture was poured into ethanol (500 ml.) and allowed to stand overnight to allow evaporation of ammonia. The starch was then filtered, and washed with ethanol. This pretreatment was given to all starches before complete dispersion experiments were carried out. This specific pretreatment probably causes expansion of the inherent water present in the granules and hence disrupts the micellar structure giving a completely disrupted granule, but causes no degradation to the components of starch (Banks et. al., 1959).

Aqueous fractionation procedures. -

(a) Aqueous dispersion. The most satisfactory method involves complete dispersion of the granules in boiling water in an inert atmosphere, followed by selective precipitation of the amylose as an insoluble complex. Complexing compounds which have been suggested for this purpose include, butan-1-ol, pentanol,

amyl alcohol, pyridine, thymol, cyclohexanol and many others. In this work thymol was used as a complexing agent and the aqueous procedure was as follows: Distilled water was boiled for 1 hour under nitrogen to remove any oxygen which may be present. To this was added a starch suspension sufficient to give ca. 0.5% solution. After boiling this solution, under nitrogen for one hour, the solution was allowed to cool to 60°C and powdered thymol (1 grm/litre) added. After standing at room temperature for three days, the amylose complex was removed on a Sharples super-centrifuge and recrystallised three times as the butan-1-ol complex. The supernatant liquid after the removal of the amylose was shaken up with ether to remove the excess thymol and after separation and removal of the ether, was freeze-dried to give amylopectin. This method ensured maximum purity and minimum degradation of the starch components.

(b) Aqueous leaching. - In order to understand more fully the fine structure of the amylose component of any starch, the amylose was subfractionated by successive leaching of the granules at 70, 80 and 90°C followed by the complete dispersion of the residue. Water was heated to the required temperature and nitrogen passed through it for one hour to remove any oxygen which may be present. A starch suspension was then added and the total solution kept at the required temperature for a further

Table 3.1.

Properties of amylose obtained by successive leaching followed by complete dispersion of granule.

Starch	Procedure	% of amylose extracted (a)	% purity (b)		$\overline{D.P.}$ (c)	β -amylolysis limit (d)
Barley	70° leach	22	101	93	690	96
	80° leach of residue	17	100	138	1000	74
	90° leach of residue	25	100	250	1800	69
	" " " "	18	100	298	2200	64
	" " " "	13	100	370	2750	63
	Dispersion of residue	5	99	380	2850	63
Iris	70° leach	19	100	190	1400	99
	80° leach of residue	25	100	230	1700	89
	90° " " "	25	100	260	1900	76
	Dispersion of residue	31	100	280	2100	72

(a) Calculated by acid hydrolysis

(b) Calculated by conversion to maltose under the concurrent action of β -amylase and Z-enzyme

(c) Calculated from the relationship $\overline{D.P.} = 7.4 \times [7]$

(d) Expressed as percentage conversion into maltose

hour. The suspension was then cooled in ice water, centrifuged and the supernatant passed through a grade 3 sintered filter. Butan-1-ol was added and the resultant amylose complex, removed by centrifugation, after standing overnight. Samples of the starch residue were removed, dried with ethanol and the amylose content determined by potentiometric iodine titration. The subfractionated amylose samples obtained in this way varied considerably both in size and in β -amylolysis limit. As the leaching temperature increased, the size of the leached amylose increased and the β -amylolysis limit decreased (see Table 3.1). Initially, attempts to obtain a linear amylose by this method failed. However linear amylose is easily obtained from methanol defatted granules. It was thought that the presence of fatty acid in the original starch granules, prevented the leaching of a linear amylose, but although potato starch contains no significant amount of fat, it requires to be refluxed with methanol before linear amylose can be obtained on leaching. The effect of boiling methanol on starch granules can therefore hardly be explained by a simple defatting action. Rather it must cause a change in the granular structure. It could be that the pretreatment weakens the micellar structure, for although microscopic examination shows the granules to be undamaged, there is a small but significant decrease in the mean gelatinisation

temperature when compared with the untreated sample. e.g.

Table 3.2

Starch	Gelatinisation temperature °C	
	Treated	Untreated
Barley	62.5	64.5
Malted Barley	66.5	67.2
Apple	56.5	57.5
Mango	75.5	77.0
Broad Bean	65.5	67.0
Iris	65.5	66.5

Moreover the methanol pretreated granules did not swell to the same extent on gelatinisation, as had the unpretreated granules. Swelling and gelatinisation are extremely complex and there can be little doubt that a change in either is probably due to alterations in the degree of order existing within the granule.

Characterisation of the fractionation products. - The amylose fraction was characterised by. -

(a) measurement of the limiting viscosity number in M-potassium hydroxide solution. The degree of polymerisation ($\overline{D.P.}$) was calculated from $\overline{D.P.} = 7.4 \times [\eta]$ which had originally been derived by Cowie and Greenwood (1957) from osmotic pressure measurements on potato amylose. It was applied to all other starch amyloses on the assumption that the error will not be large (Arbuckle and Greenwood 1958).

(b) measurement of iodine affinity by potentiometric titration. Under the conditions employed, the iodine affinity of the amyloses varied from 19.0 in the cereal amyloses to 19.9 in banana amylose

(c) determination of the β-amylolysis limit using purified soya-bean β-amylase at pH. 4.6 (Peat, Pirt and Whelan 1952b).

(d) determination of phosphorus content using the method of Fogg and Wilkinson (1958).

The amylopectin fraction was characterised by. -

(a) measurement of limiting viscosity in 1M-potassium hydroxide.

(b) determination of β-amylolysis limit as above

(c) determination of purity by measurement of iodine affinity

(d) average unit-chain length, by oxidation with aqueous sodium periodate

(e) determination of the sedimentation velocity (S_{20}) in water

(f) determination of the molecular weight by light scattering techniques kindly carried out by Dr. W. Banks

3d

Fractionation in an inert atmosphere

All starch fractionations were carried out in a nitrogen atmosphere. This was essential because barriers can be introduced into amylose by oxygen. In 1956, Baum, Gilbert and Scott produced evidence of this fact. They found that amylose heated in a stream of oxygen in neutral or alkaline solution changed its nature; whereas the original amylose was hydrolysed by phosphorylase to 90%, the oxygen-treated amylose had a phosphorylase limit of 70-80%. According to Baum et. al., this treatment had introduced barriers to phosphorylase and by analogy to β -amylase action. However Cowie et.al., in 1957 treated amylose in 0.5 M-sodium hydroxide, with oxygen for 20 minutes and found that the β -amylolysis limit increased. They therefore concluded that alkali-oxygen treatment did not introduce barriers to β -amylase.

It is not however possible to relate changes in the β -amylolysis limit to the effect of alkaline treatment, without considering the changes which must occur in the molecular size of the amylose during this treatment. Hydrolytic or degradative action occurring concurrently with the introduction of a barrier or artefact would naturally mask the effect of this barrier on the β -amylolysis limit. As Cowie et. al. (1957)

had not carried out viscosity measurements their results were inconclusive.

In order to check the possible introduction of barriers into amylose by oxygen a series of experiments were carried out on linear amyloses obtained from defatted potato and iris starch.

Oxygenation procedure. - Approximately 50 mg of amylose was dissolved in 20 ml. 0.2 M-potassium hydroxide, and the pH. adjusted to 9.2 with 1M-acetic acid. The flask containing the solution was placed in a boiling water bath and oxygen bubbled through for 20 mins. The amylose was then precipitated as the butan-1-ol complex and centrifuged off. A control experiment using iris amylose in a nitrogen atmosphere and the same solvent, was also carried out. Each amylose was determined by measurement of (a) the limiting viscosity number in 1M KOH (b) the β -amylolysis limit and (c) the concurrent effect of β -amylase and Z-enzyme.

Results and discussion. - Table 3.3 shows the results of these experiments. As linear amylose was used in the oxygenation treatment, it is obvious from the viscosity and β -amylolysis limits that barriers have been introduced into the amylose by the oxygen. Only in the control experiment where nitrogen was used instead of oxygen, did the β -amylolysis limit and the limiting viscosity number show no appreciable change. This therefore confirms, that it is the presence of molecular oxygen that is introducing the barrier. This is further supported by the fact

Table 3.3

Effect of oxygen at 95° on linear amylose fractions

Amylose [a]	Conditions pH. [b]	time (mins)	Initial	Final	β-limits (1)	(11)
Potato 1	9.2	20	290	260	86	100
Potato 2	9.2	20	260	220	86	100
Potato 3	9.2	20	230	195	87	100
Iris germanica 1	9.2	20	190	120	86	100
" " "	9.2+	20	190	180	98	100
" " "	7.0	20	190	130	91	100
" " "	Water	120	190	160	95	100

a Potato 1 = Redskin; Potato 2 = Golden Wonder; Potato 3 = Homeguard

b Buffered solutions

c Expressed as percentage conversion to maltose (1) β-amylase

(2) β-amylase + Z-enzyme

\+ Control run with N_2-atmosphere

that in neutral solution both the β -amylolysis limit and limiting viscosity number decreased.

The fact that barriers are introduced in neutral solution suggested that a similar process might occur during the fractionation of starch. Confirmation of this occurrence was obtained by Banks (1960) who fractionated starches in the presence of air, oxygen and nitrogen. During the isolation of the amylose from the starches in the presence of oxygen or air appreciable degradation occurred which was shown by the decrease in limiting viscosity number. At the same time however the β -amylolysis limit also decreased, thus suggesting that the presence of oxygen during fractionation did, in fact, introduce barriers to the action of β -amylase.

3e

Fractionation Of Potato Starch By Centrifugation In Alkali.

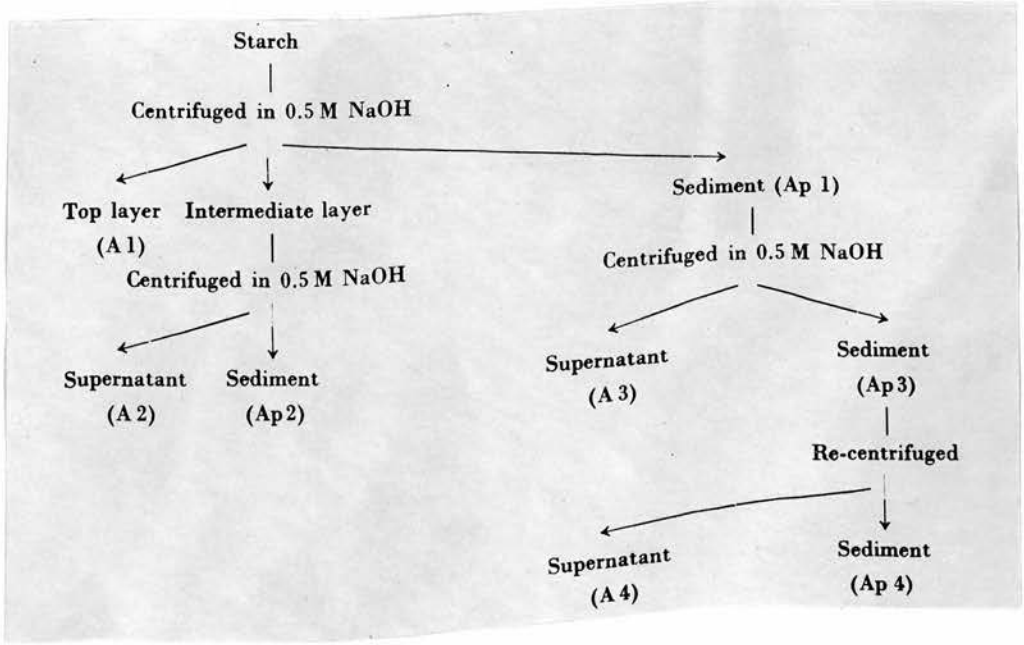
It was reported by Baum and Gilbert in 1956 that potato starch could be fractionated into its separate components by the centrifugation of dispersions of undamaged granules in dilute alkali. According to these workers, the amylopectin component was completely insoluble in this medium and the amylose component completely soluble, hence on centrifugation under a force field of 40,000 g. for two hours, the two components could be easily separated. As the limiting viscosity number of the amylose and amylopectin obtained, was similar to that obtained under normal fractionation procedures the authors concluded that this was conclusive evidence of the fact that amylose and amylopectin are separate entities and are not joined together by covalent bonds in the starch granule.

This facet of starch chemistry is obviously of primary importance and merits careful study. The experimental procedure was therefore repeated on redskin potato granules using the preparative-rotor of the "Spinco" model E ultracentrifuge and careful characterisation of the separated components was carried out,

Experimental procedure. - Starch was stirred at 0.6% concentration in 0.5M sodium hydroxide under nitrogen and at room temperature for 30 mins. After this time the gelatinized granules were removed by

centrifugation in polythene tubes at a mean force field of 40,000 g. for 2 hours in the preparative rotor of the Spinco ultracentrifuge. The top one-third of each tube was then removed by pipette, the solution carefully neutralised and the amylose precipitated by the addition of butan-1-ol. The residual liquor and the sediment of gelatinized material was then re-extracted with alkali as indicated in Figure 3.1., where amylose containing supernatants are labelled (A) and amylopectin containing sediments are labelled (Ap). The whole leaching experiment was repeated using a mean force-field of 20,000 g.

Figure 3.1



Results and discussion. - The properties of the various fractions obtained by this method are shown in Table 3.4. The overall yield amounted to 90% for Experiment I and 83% for II; losses were primarily in the amylopectin as the total of amylose in the different fractions accounted for about 20% of the original weight of starch. The yield of amylose in fraction A 1 (1) was about 60% for Experiment I and 80% for Experiment II. The initial precipitates were very impure (64 and 44% amylose respectively), but on recrystallisation twice as the butan-1-ol complex, amylose was obtained with a limiting viscosity number $[\eta]$ of 435-470 compared to that of an amylose from a conventional dispersion of this starch (i.e. 480). Further the alkaline treatment had not altered the β-amylolysis limit of about 85%. These amyloses represent a portion of the total amylose present in the granule. Fractions A3 and A4, which precipitated on neutralisation of the alkaline solution before the addition of butan-1-ol, were mainly amylopectin and the two re-extractions of Ap 1 therefore would appear to have resulted simply in solution of amylopectin. Recovery of amylopectin in the sediments was poor being only about 50% for Experiment I and 30% for II. Further, the purity (2-7% of amylose) was not comparable to that of amylopectin from a conventional dispersion when 0.5-1% of amylose

Table 3.4

Characteristics of products from centrifugal fractionation of starch in alkali

Exp.	I (at 40,000 g. force field				II (at 20,000 force field			
Fraction	Weight in (mgs)	% of a. amylose	[η]	β - b. -limit	Weight in (mgs)	% of a. amylose	[η]	β - b. -limit
A 1 (1) c.	250	64	200	78	460	44	215	74
A 1 (11) c.	-	100	470	86	-	100	435	85
A 2	140	40	214	68	68	35	185	71
A 3	100	7	185	58	92	6	220	60
A 4	60	5	180	60	58	4	205	58
Ap 1	not isolated		-	-	not isolated		-	-
Ap 2	90	7	210	58	110	7	185	59
Ap 3	not isolated		-	-	not isolated		-	-
Ap 4	450	4	215	57	190	2	180	57

a. Calculated from (iodine affinity ÷ 19.5) x 100

b. percentage conversion to maltose by β -amylase

c. A 1 (1) = sample before recrystallisation

A 1 (11) = sample after recrystallisation

is usual.

On the basis of these results, fractionation by this method is unsatisfactory. Although separation can be achieved it is not good. However it is obvious that there are no bonds between amylose and amylopectin which resist cold alkali.

The results imply that either (1) amylopectin is insoluble in alkali and amylose is leached from the granule, or (2) solution is achieved and the amylopectin undergoes simple preferential sedimentation. Whereas Baum and Gilbert (1956), favour the idea of amylopectin insolubility, the fact that amylose A 1 (11) has the properties of a total amylose, which can only be obtained by complete granular dispersion means that complete solution of the granule has occurred and the components are sedimenting in the force-field used.

3f

Subfractionation Of Amylose In Dimethyl Sulphoxide Solution.

Several attempts to fractionate amylose have been reported and these were critically examined by Jones (1959). The precipitation of amylose from aqueous solution by ethanol yielded insoluble products, and attempted fraction - ation from ethylenediamine by the same method were no more successful. Lansky, Kooi and Schoch (1949) reported the fractionation of amylose using n-octyl alcohol as a pre- cipitant. However as their results showed that in all cases the average viscosity of the fractions was considerably less than that of the parent amylose, the fractionation must have been accompanied by degradation. Recently, Hollo and Szejtli (1958) used the iodine-amylose complexing method of Rundle et. al. (1944) to fractionate amylose. This method was found by Jones (1959) to be unsuccessful as no fraction having a viscosity greater than the parent amylose was obtained. Goodison and Higginbotham (1950) fractionated amylose by slowly cooling the amylose-butan-1-ol complex. The solubility of this complex is dependant on temperature and molecular size although the danger of degradation at the high temperatures employed is very great especially as it is almost impossible to keep the atmosphere during fractionation

oxygen free.

The most promising method of fractionation was that of Everett and Foster (1959) who fractionated amylose by precipitation from dimethyl sulphoxide with ethanol. From their viscosity and light scattering results they concluded that amylose behaves as a random coil in solution. Dimethyl sulphoxide has been used as a method of starch granule pre-treatment, and results have shown that no degradation of the starch fractions occur. This procedure was therefore investigated in detail.

Isolation of amylose. - Amylose was isolated from Iris starch by aqueous dispersion of the granules. The amylose was purified by the usual method of recrystallisation from butan-1-ol and then dried *in vacuo* at 80°C.

Method of fractionation. - Amylose was dissolved in dimethyl sulphoxide at room temperature to give a solution having a concentration of *ca.* 0.5%. Ethanol was added to a concentration of 20% (by volume) and the mixture cooled to 4°C in an ice-bath. Alcohol was added dropwise to the cold solution, with vigorous stirring until the solution became slightly turbid. This precipitate was removed by centrifugation. The gel-precipitate was fully precipitated with ethanol and filtered off. The precipitation process was repeated until eight separate fractions had been obtained and each fraction

Table 3.5

Subfractionation, sedimentation coefficient and purity of the Iris Amylose fractions.

Fraction	% Alcohol	% amylose pptd.	Sedimentation Velocity	% Purity
1	41.2	1.90	14.6	99
2a	41.2	3.50	11.2	"
2b	42.8	2.60	10.4	"
3a	43.9	2.10	11.0	"
3b	45.2	31.50	10.6	"
3c	46.8	9.40	9.6	"
4a	47.5	11.40	9.7	"
4b	48.2	10.40	7.2	"
5	48.2	10.40	7.4	"
6	50.0	11.40	6.8	"
7	52.4	3.50	5.1	"
8	56.4	1.90	4.4	"

Table 3.6

Limiting viscosity numbers, degree of polymerisation and β-amylolysis limits of the Iris amylose subfractions.

Fraction	KCl $[\eta]$	KOH $[\eta]$	D.P. a	β-amylolysis limit b
1	330	540	4000	62
2a	270	410	3000	65
2b	255	360	2700	100
3a	270	380	2800	70
3b	230	320	2400	88
3c	200	260	1900	100
4a	210	280	2100	86
4b	130	150	1100	92
5	100	130	1000	95
6	95	110	800	95
7	80	85	600	100
8	75	80	600	100

a. From relationship $\overline{D.P.} = 7.4 \times [\eta]$

b. percentage conversion into maltose by β-amylase

was washed free from dimethyl sulphoxide and weighed. Fractions 2,3 and 4 were refractionated giving 12 fractions in all. A precipitation curve for the fractionation of iris amylose is shown in Figure 3.2.

Characterisation of the amylose fractions. - Each amylose fraction was characterised by the following procedures

(a) Viscosity. The limiting viscosity for each fraction was measured in both 0.2 M KOH and 0.33 M-KCl.

(b) Sedimentation. The sedimentation coefficient was measured in $0.33_{\Lambda}^{\bar{M}}$ KCl solution. The sedimentation coefficient at infinite dilution was obtained by extrapolating from the plot S^{-1} versus concentration.

(c) Enzymic hydrolysis limits. The β-amylolysis limit of each amylose fraction was determined and the purity of the fractions estimated by the concurrent action of β-amylase and Z-enzyme. For results see Tables 3.5 and 3.6.

Discussion of results. - As can be seen from β-amylolysis limits and limiting viscosity measurements, this method is highly successful and good fractionation of amylose occurs. The efficiency of the fractionation was clearly shown by comparing the properties (limiting viscosity number in KOH and KCl, and β-amylolysis limits) of the parent amyloses with the corresponding calculated values. Table 3.7 gives the comparative results.

FIG. 3.2

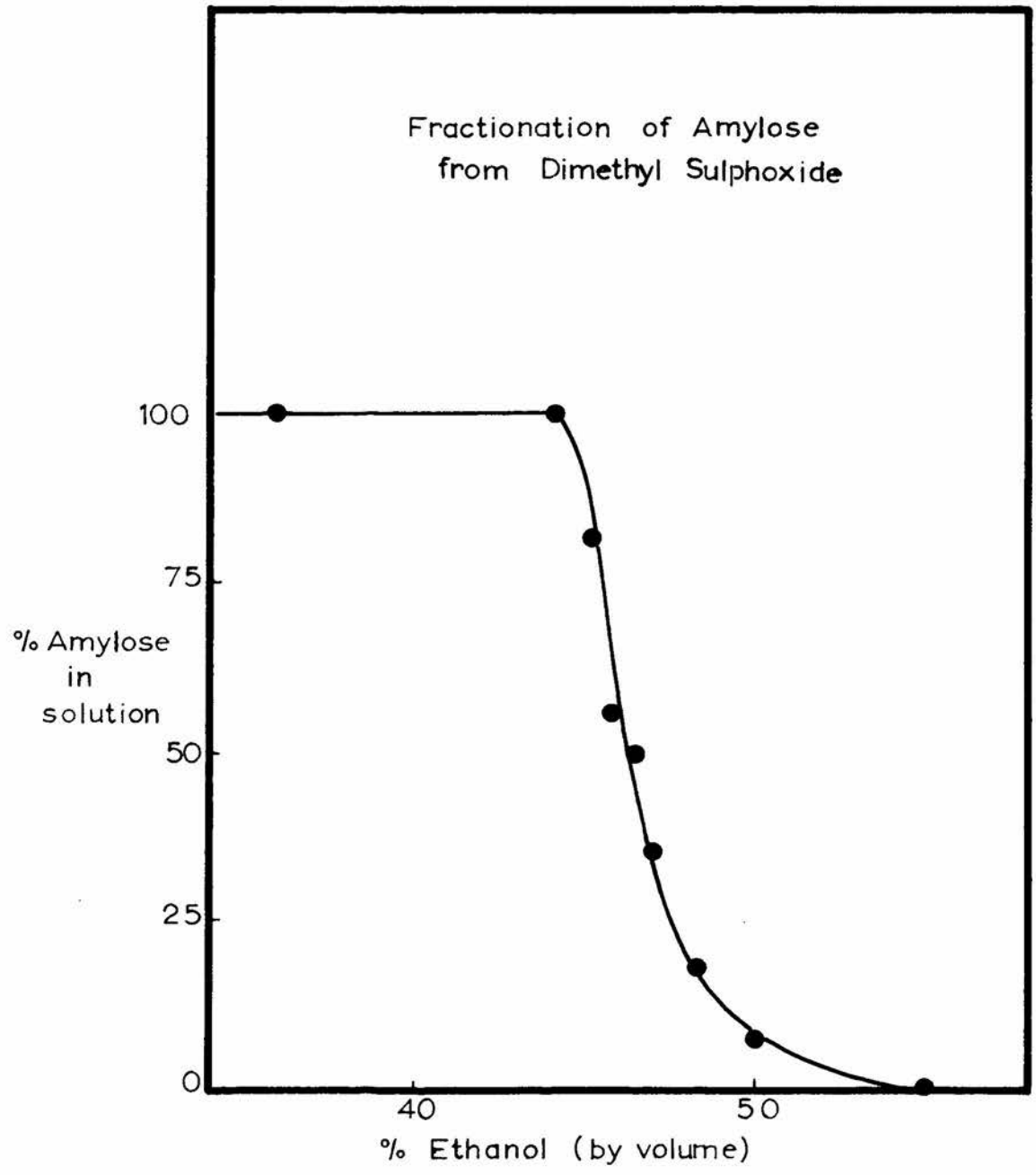

Fractionation of Amylose
from Dimethyl Sulphoxide
100
75
50
25
0
% Amylose
in
solution
40
50
% Ethanol (by volume)

Table 3.7

	$[\eta]$ KCl	$[\eta]$ KOH	β -limit
Experimental Value	175	240	84.6
Calculated Value	185	240	86.2

There is remarkably good correlation between the calculated and the experimental values, showing that fractionation has been achieved without degradation occurring.

Section 4

The Molecular Properties of the Components of Starches.

4a.

Fruit Starches

Starch was obtained from apple, banana and mango fruits by the extraction method described in section 3a. The starch present in fruits is converted rapidly to fructose and glucose when the fruit ripens. Apples and bananas contain in the unripe state about 20% and 60% starch respectively, but as they ripen the starch content of both fruits falls to about 5%. Mango starch obtained from the seed in the centre of the fruit presented an unusual purification problem. There was present in the seed a considerable amount of iron. As inorganic elements like iron are known to interfere with enzyme actions, it had to be removed before β -amylolysis limits could be carried out on the starch components. The most suitable time to remove the iron was when the starch was in the granule state and several washes with 0.1 N-hydrochloric acid followed by careful washing with water successfully removed the impurity.

All the fruit starches were found to contain considerable amounts (5%) of fibrous material, which could not be removed from the granules by filtration or centrifugation. However after conventional dispersion (as in section 3b), the fibre appeared as a fine layer in the Sharples super-centrifuge and could be easily removed.

Characterisation of the Starches:- The starches were characterised by determination of (a) purity (b) gelatinisation temperature (c) iodine affinity (d) size distribution of the granules (see section 3).

Table 4.01

The Characteristics Of The Fruit Starches.

urce of arch	Shape of Granule	Average size of Granule (μ)	Protein in Starch (%) [a]	Iodine Affinity (I. A.) [b]	Gelatinisation Temperature °C
ple	Round	5-10	0.100	3.60	55-57
nana	Pear Shaped	35	0.325	3.00	68-70
ngo	Oval	27	0.250	4.45	74-77

a Calculated by % Nitrogen x 6.25

b Amylose content obtained from $\frac{\text{Iodine affinity of Starch}}{\text{Iodine affinity of amylose}} \times 100$

Table 4.02

General characteristics of the amylose component of fruit starches. -

Amylose	[η] in M-KOH	D.P. [a]	Iodine Affinity [b]	Conversion limits (1) [c]	(2) [c]
Apple	200	1500	19.0	84	100
Banana	230	1700	19.9	82	100
Mango	240	1800	19.2	77	100

a D.P. = degree of polimerisation calculated by D.P. = 7.4 x [η]

b By potentiometric titration

c Expressed as percentage conversion into maltose under the action of (1) β -amylase and (2) β -amylase + Z-enzyme.

Table 4.03

General characteristics of the amylopectin component of fruit starches.

Amylopectin	I.A.[a]	[η] in KOH	β-limit[b]	C.L.[c]	% P.[d]	S_{20}[e] (Svedbergs)	$D_m \times 10^7$[f]	M.W. $\times 10^{-6}$[g]
Apple	0.18	170	58	23	0.025	280	0.468	1000
Banana	0.30	120	59	21	0.020	300	0.492	220
Mango	0.32	150	56	21	0.012	250	0.444	425

a IA = Iodine affinity

b expressed as % conversion to maltose

c Calculated by oxidation with sodium metaperiodate

d As in section 2.k.

e S_{20} = sedimentation velocity at 20° in water.

f as in section 2.f.

g M.W. = Molecular weight obtained from light scattering results carried out by Dr. Banks.

The starch components obtained by convensional dispersion of the granules and by combined aqueous leaching and conventional dispersion (as in section 3) were characterised by determination of -

Amylose fractions - (a) limiting viscosity number $[\eta]$ (b) β -amylolysis limit (c) iodine affinity.

Amylopectin fractions - (a) limiting viscosity number (b) β -amylolysis limit (c) iodine affinity (d) unit chain-length (e) phosphorus content (f) sedimentation velocity (g) diffusion coefficient (h) molecular weight.

Results and discussion - These fruit starches have not been studied by other workers to the same extent as root and tuber starches. The relative expense and the seasonal changes in the starch content are factors effecting their commercial value as a source of starch. As can be seen from a comparison of results, the amylose fraction of the fruit starches is comparable with the amylose from cereal starches but considerably smaller than the amylose of the root and tuber starches. It is of interest to note that the molecular weight (Mw) of the fruit starch amylopectins is considerably higher than most of the other starches studied.

Root, Rhizome And Tuber Starch.

Roots, rhizomes and tubers provide the most abundant sources of starch. In many botanical families, these are the primary storage organs in which plants deposit their reserve carbohydrates. Starch can be easily obtained from these sources by normal extraction methods (as in section 3a), and are obtained in a very pure state i.e. free from proteinous or fibrous material.

In this study starches were obtained from the rhizomes of Iris Germanica, the roots of the parsnip and the tubers of the potato (var. Redskin).

These starches were fractionated by the conventional dispersion method described in section 3b and the separated components characterised.

Discussion. - There is a general similarity between the amylopectin fractions of these starches. Their β -amylolysis limits, chain-lengths, sedimentation velocities and diffusion constants are all similar. Light scattering results however show that the weight average molecular weight of parsnip amylopectin (400×10^6) is considerably greater than the corresponding molecular weight of iris (90×10^6) or potato (65×10^6).

The amylose fractions are similar with regard to β -amylolysis limit and iodine affinity but differ considerably in size. The amylose fractions obtained from the potato and parsnip starches have a very large $\overline{D.P.}$ of 3400 and 4300 glucose units respectively. These are considerably bigger than any other amylose obtained from other

Table 4.04.

The general characteristics of Iris, Parsnip and Potato starch.

Source of starch	Shape of Granules	Average size of granules (μ)	Protein[a] in starch (%)	I.A.[b]	Gelatinisation Temperature
Iris	Oval	30	0.075	5.00	64-67
Parsnip	Round	10-15	0.275	2.10	53-55
Potato	Round	40	0.100	4.30	62-65

a % Protein = % Nitrogen x 6.25

b I.A. = Iodine affinity

Table 4.05.

General characteristics of the amylose component of Iris, Parsnip and Potato starch

Amylose	$[\eta]$ in M-KOH	D.P. [a]	I.A. [b]	Conversion Limits [c] (1)	(2)
Iris	240	1800	19.10	84	100
Parsnip	590	4300	19.40	72	100
Potato	460	3400	19.20	84	100

a D.P. = 7.4 x

b I.A. = Iodine Affinity

c % conversion to maltose by the action of (1) β -amylase

(2) β -amylase + Z-enzyme

Table 4.06

General characteristics of the amylopectin component

of Iris, Parsnip and Potato starch

Amylopectin	I.A. [a]	$[\eta]$ in KOH	β-limit [b]	C.L. [c]	% P. [d]	S_{20} (Svedbergs) [e]	$D_m \times 10^7$ [f]	M.W. $\times 10^{-6}$ [g]
Iris	0.18	100	57	22	0.048	300	0.480	90
Parsnip	0.16	175	58	21	0.054	320	0.350	400
Potato	0.14	160	57	24	0.044	350	0.580	65

a to g as in Table 4.03

botanical sources. However it is extremely interesting to note that the amylopectin components of these starches have an unusually high phosphorus content and this fact may give some support to Erlanders theory of starch synthesis (see Section 1).

4c.

Seed Starches.

Starch is stored in the seed of a plant, to act as the reserve polysaccharide, which is used to aid the growth of the young seedling.

Seed starches are easily obtained by the method given in section 3, but are difficult to purify and require several extractions with toluene to remove the large amount of protein present. The sample of amylomaize starch (high-amylose maize starch) was kindly donated by Dr. F.R. Senti.

Results and discussion. - The components of the seed starches are very similar to one another. Table 4.08 gives the characteristics of the amylose fractions, which are very similar in size and extent of enzymic attack, while Table 4.09 indicates considerable similarity in the size of the amylopectin fractions. Amylomaize starch is exceptional, in that there is 52.3% amylose present (IA = 9.92). This starch had a high gelatinisation temperature (85-87°C) and on conventional dispersion of the ammonia pretreated starch, yielded a crude amylopectin component (amylopectin (1)). This contained a considerable amount of very small amylose which could only be removed by differential ultracentrifugation. The amylopectin obtained by this method amylopectin (2) was comparable in size to the amylopectins from the other seed starches (Table 4.09). The small amylose obtained was linear by nature (β-limit 100%), and had a D.P. of 250. A starch of the nature of amylomaize starch, is of considerable commercial importance, as a good, easily accessible source of amylose.

Table 4.07

General characteristics of Rubber Seed, Broad Bean and Amylomaize starches.

Source of Starch	Shape of Granule	Average size of granule (μ.)	Protein in starch [a]	I.A. [b]	Gelatinisation Temperature
Rubber Seed	Round	10	0.156	3.70	67-69
Broad Bean	Oval	30	0.110	4.45	64-67
Amylomaize	Round	25	0.274	9.92	85-87

[a] As in Table 4.01

[b] IA = Iodine Affinity

Table 4.08

General characteristics of the amylose component of Rubber Seed, Broad Bean and Amylomaize starch

Amylose	$[\eta]$ in M-KOH	D.P. [a]	I.A. [b]	Conversion Limits [c] (1)	(2)
Rubber Seed	200	1500	19.00	74	100
Broad Bean	240	1800	19.20	82	100
Amylomaize	180	1300	19.20	77	100

a, b and c As in Table 4.02

Table 4.09.

General characteristics of the amylopectin component of Rubber seed, Broad Bean and Amylomaize starch.

Amylopectin	I.A.[a]	$[\eta]$ in KOH	β-limit[b]	C.L.[c]	(%) P.[d]	S_{20} (Svedbergs)[e]	D^m x 10^7 [f]	M.W.[g]
Rubber Seed	0.28	140	58	23	0.022	260	0.462	-
Broad Bean	0.36	125	58	23	0.017	280	0.500	450
Amylomaize (1)	-	-	64	36	0.026	-	-	-
Amylomaize (2)	0.36	130	58	28	0.030	270	0.480	-

a - g as in Table 4.03

(1) Amylopectin before the removal of the small amylose.

(2) Characteristics of amylopectin after the removal of the small amylose by ultracentrifugation.

4d.

Floridean Starch

Introduction

Granular material which gives a colour with iodine was first observed in various red algæ, when sections of the latter were observed under the microscope. This fact was first reported as far back as 1865 by Nägeli. The colour with iodine was not the characteristic blue, shown by normal starches but varied from deep violet to brown. It was attributed to a polysaccharide designated "Floridean" starch, the structure of which has been considerably investigated.

Early studies by Colin et. al. (1933) showed, that not only did the polysaccharide stain violet with iodine, but also that it was dextro rotatory. Kylin (1913), found that the glucosan, obtained from the red algæ, Dilsea edulis, could be degraded by dialysed malt extract to maltose. Barry, Halsall, Hirst and Jones (1949), examined another sample of Floridean starch isolated from Dilsea edulis. They found it to be a glucosan, $[\alpha]_D$ + 156 in water, which according to their experiments resisted the attack of sweet potato β -amylase. On oxidation with sodium metaperiodate, one molecule of formic acid was liberated per 18 glucose residues.

Another sample of Floridean starch was investigated by O'Colla. His material which was contaminated by a galactan sulphate (18%) had an $[\alpha]_D$ + 166 in water and gave 50% of maltose on treatment with wheat β -amylase. On periodate oxidation, one molecule of formic acid was liberated per 12 glucose residues. Fleming et. al. (1956) re-examined the sample of Floridean starch originally studied by

Barry et. al. (1949) and found that the glucosan had an $[\alpha]_D$ + 176 in water a β -amylolysis limit of 46% and gave on periodate oxidation a unit-chain length of 9 glucose units. Peat, Turvey and Evans (1959), isolated Floridean starch from yet another sample of Dilsea edulis. Their study was very meticulous and they showed that Floridean starch possessed the ramified structure previously reported by Barry et. al. (1949) in which the main chain links were α - 1:4 -glucosidic and the branch-links α - 1:6 as in amylopectin and glycogen. On partial acid hydrolysis nigerose was isolated chromatographically. This strongly indicated the presence of some α - 1:3 linkages. The sample had an $[\alpha]_D$ + 173 in water, a β -amylolysis limit of 42% and on periodate oxidation one mol.of formic acid was liberated for every 15 glucose units.

Floridean starch was obviously an unusual starch. It apparently contained no amylose and was of the glycogen or amylopectin type. Its actual structure has not however been established conclusively, as can be seen from the variety of conflicting results in Table 4.10.

It was decided to carry out a comparative study of the structure of Floridean starch with other related branched α - 1:4-glucans in order to establish initially, whether the polysaccharide was more related to plant amylopectins or animal glycogens. In order to fully study the glucan, it was isolated and purified in granular and non-granular form from a sample of Dilsea edulis. The difficulties

Table 4.10

Comparison results for the characterisation of floridean starch

	Authors			
	Barry et. al.	O'Colla	Fleming et. al.	Peat et. al.
$[\alpha]_D$ in water	156	166	176	173
β -amylolysis limit [a]	3	50	46	42
Average length of unit chain [b]	18	12	9	15

a Using purified soya-bean β -amylase

b by oxidation with sodium metaperiodate

encountered in doing this were not unexpected. Aqueous extracts of marine red-algae always contain a complex mixture of polysaccharides. All previous workers in this field, found great difficulty in purifying the Floridean Starch, free from the contaminating galactan sulphate. Previously only chemical methods of purification had been attempted, not only had these proved to be unsuccessful but they may also have considerably degraded the polysaccharide.

Experimental. - (a) Granular starch.

A small quantity of granular material was isolated from the fronds of Dilsea edulis by extraction of M/100 mercuric chloride, in a blendor. The extract was filtered through muslin and the granular material obtained by centrifugation. The gelatinisation temperature of these granules in water was 45-47°C but the procedure was not efficient and other methods had to be used.

(b) non-granular starch.

About 250 grm of macerated fronds were steeped in water under toluene at 4°C for four days to remove mucilaginous material. The fronds were then pretreated with liquid ammonia (as in section 3). The pre-treated fronds were then dispersed in water under nitrogen at 98°C. Insoluble material was removed by centrifugation, calcium chloride (1 vol. of saturated solution), was added and the mixture left at 2°C for 24 hours. Precipitated calcium salts were removed by centrifugation, the solution dialysed against distilled water and the Floridean Starch isolated by freeze-drying the dialysed solution.

This product contained a galactan impurity as shown by chromatographic examination of the hydrolysate. This was removed by the purely physical process of three successive differential ultracentrifugations of an 0.5% aqueous solution at 40,000 r.p.m. (100,000 g.), in the preparative rotor of the "Spinco" model E. ultracentrifuge. The final product gave on hydrolysis only glucose and contained 98.5% of reducing sugar as estimated by the alkaline-ferricyanide titration.

In order to characterise the starch, measurements were made of (1) the iodine affinity (2) the optical density of the glucan-iodine complex at various wave lengths (3) the β-amylolysis limit (4) the average length of unit-chain by oxidation with sodium metaperiodate (5) the sedimentation coefficient in 0.2M potassium hydroxide (6) the limiting viscosity number in 1M potassium hydroxide (7) the molecular weight of the glucan (M_w) from light scattering measurements (The latter was kindly provided by Dr. W. Banks). The fully characterised starch was then critically compared with other, possibly related, glucans of the α-1:4 type.

Results and discussion

The fact that a granular material exhibiting birefringent properties was isolated suggests that this amylaceous polysaccharide is, in fact a starch. However, the crystallinity and degree of order in the granule must be radically different from that of the normal starch, as the gelatinisation temperature (45-47°) is much lower than is usual for starches (60-80°C). This is probably related to

the different conditions of botanical environment.

The method of purification of the non-granular starch, would remove any trace of short-chained amyloses which might be present. However, as the supernatant liquor after ultracentrifugation did not give an iodine-stain, it is not likely that any amylose is present.

Table 4.11 compares the characteristics found for Floridean Starch with these of a typical animal glycogen (from rabbit liver) and a typical amylopectin (from potato). There is also included in the table comparative results for the water soluble polysaccharide from sweet corn (Zea-mays polysaccharide) and from malted-barley amylopectin.

The limit of β -amylolysis for the Floridean Starch is similar to those reported by other workers (see Table 4.10). The value of 18.6 anhydroglucose units for the average length of unit-chain length was similar to that reported by Peat et. al. (1959), but differed from the value obtained by Fleming et. al. (1956). These results were not in themselves completely indicative of the type of structure. It was noted however that the amylopectin of malted barley starch and floridean starch itself had similar properties. Calculation of internal chain-length showed that the glycogen and Zea-mays polysaccharide studied had similar internal chain-lengths of 4-5 glucose units, while the amylopectins and floridean starch had internal chain-lengths of 7-8 glucose units. It was also apparent that the limiting viscosity values for glycogen and Zea-mays polysaccharide while being similar to

Table 4.11

Physical properties of Floridean starch and some other glucans.

	Glycogen	Zea-mays polysaccharide	Floridean Starch	Potato Amylopectin	Malted-barley Amylopectin
λ max.	480	500	550	550	550
β -limit	45	49	49	56	48
[?] in KOH	7	7	160	200	150
Chain length	13	13	18.6	24	18.4
Internal C.L.	5	5	7	8	7
Molecular Weight x 10^6		-	700		-

one another were considerably different from the other samples studied. These differences were emphasised by the behaviour of the various glucans towards iodine. As is shown in Figure 4.1, the glycogen and Zea-mays polysaccharides both had similar, low iodine binding powers, while the other glucans studied had a pronouncedly higher affinity for iodine.

A further essential difference between branched glucan structures is their behaviour on ultracentrifugation in alkaline solution. The sedimentation coefficient for glycogen type materials is relatively independent of concentration whilst amylopectins are highly concentration dependent. Figure 4.2 shows the results for the different glucans and as before only the glycogen and Zea-mays polysaccharide had sedimentation velocities little effected by concentration, all the other including floridean starch being very concentration dependent.

The above evidence suggests that this sample of pure floridean starch, behaves as though it had an amylopectin-type rather than glycogen-type structure. This is supported indirectly by the enzymic degradation experiments of Peat et. al. (1959), who showed that their sample of floridean starch was degraded with R-enzyme. Further evidence supporting this conclusion was by light scattering experiments. By this method the molecular weight (M_w) of floridean starch was found to be 7×10^8 which is comparable with other amylopectins and considerably greater than any glycogen.

FIG. 4.1

The iodine binding power of branched α-1-4 glucans

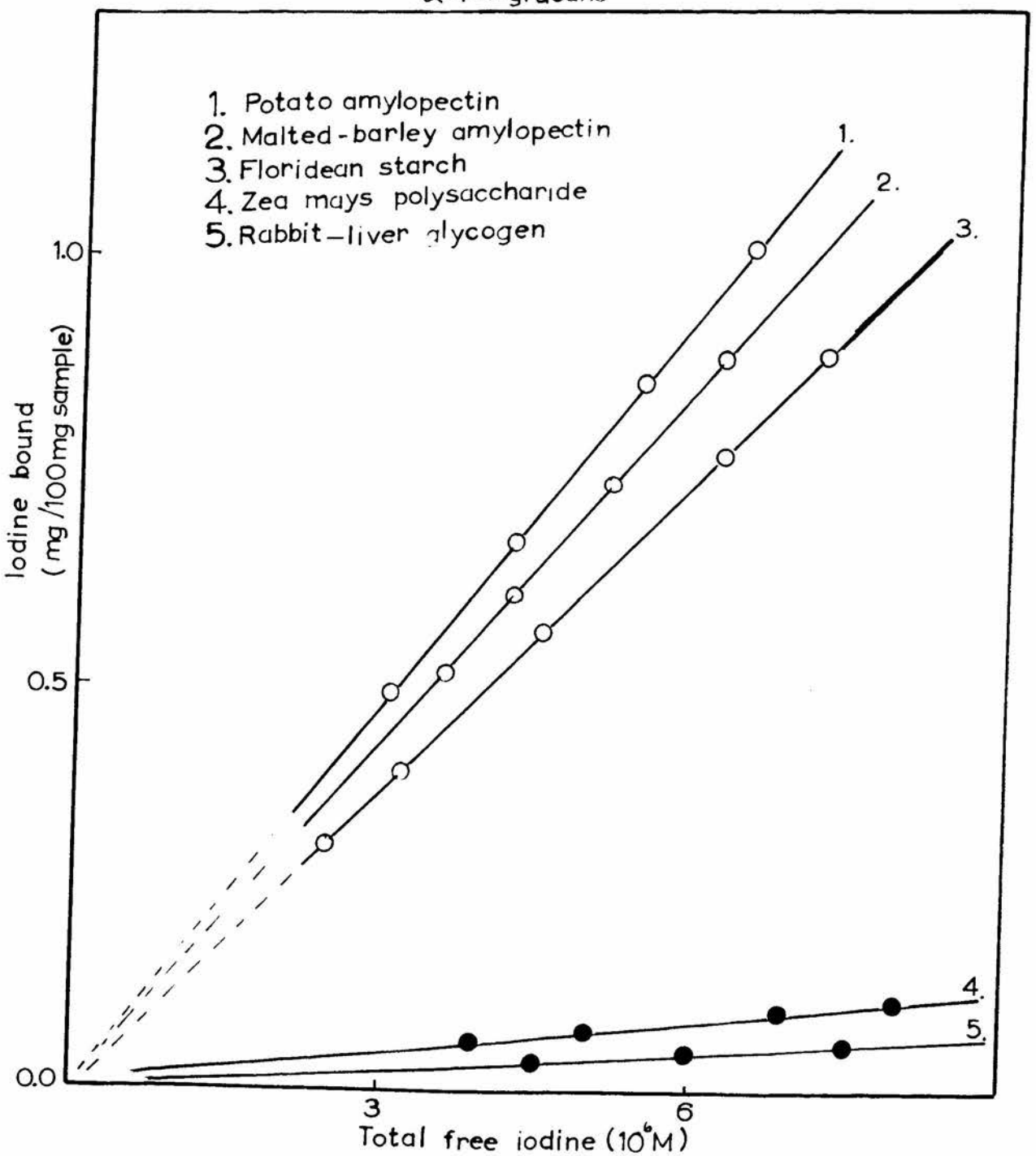
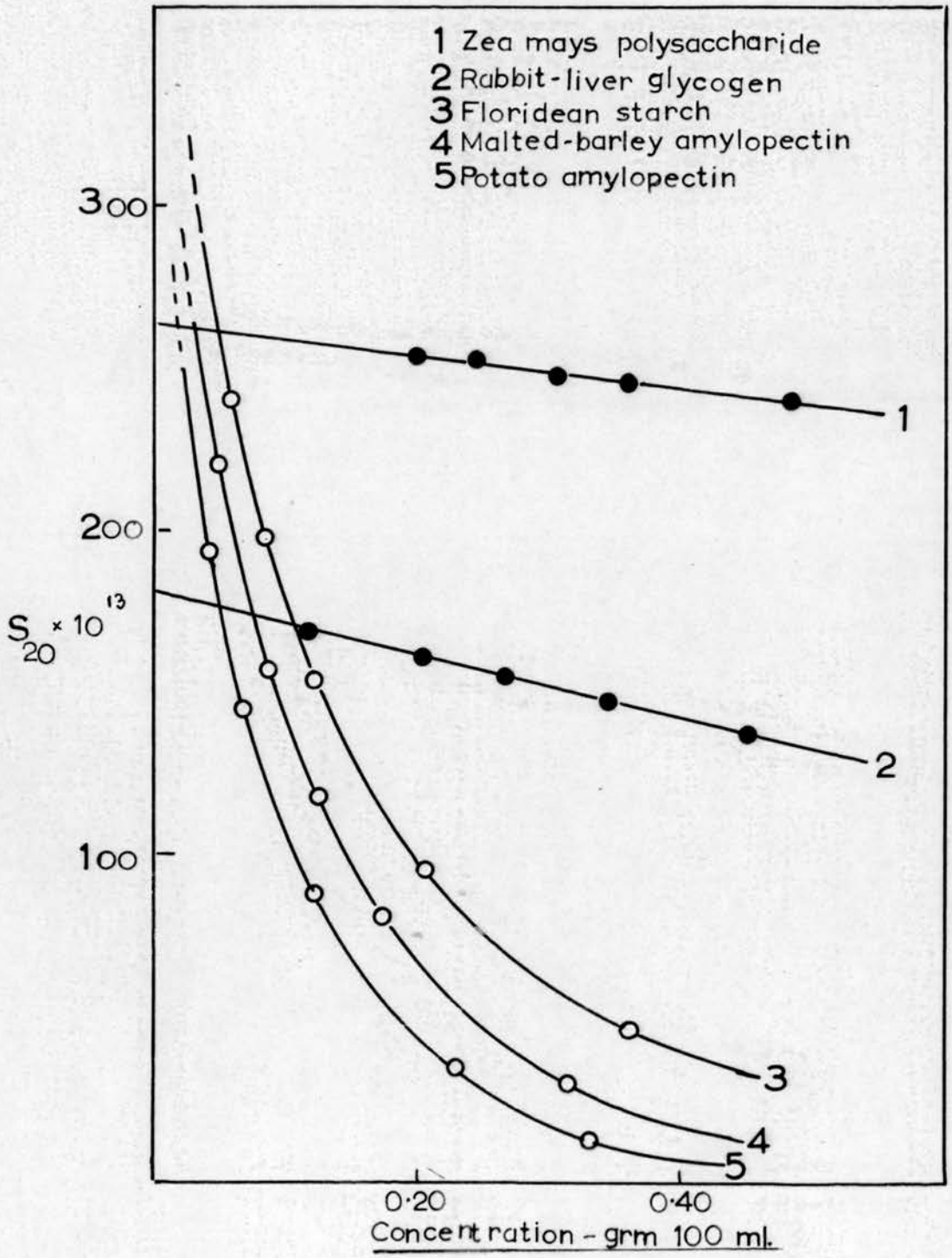


FIG. 4·2

Sedimentation behaviour



4e.

A Comparison Of The Starches From Barley And Malted Barley

Introduction

In order to understand more fully the pattern of enzymic action involved in malting, a comparative study of the starches from Ymer barley and malted barley was made. Starch was isolated from the kernals of barley and malted barley and purified free from protein impurity by the methods described in section 3. In view of the considerable analysis and resynthesis of the starch during the malting process, it was interesting to note, that the percentage starch present in the malted barley kernals was less than that in the original barley. The loss represented about 16% of the initial weight of starch (cf. 18% found by Hall et. al. (1956).

Estimation of starch in kernals. In order to estimate the starch present in the kernels the procedure of McWilliam et. al. (1956), in which the starch was removed from the kernal by dispersion with perchloric acid. The extracted starch was then precipitated quantitatively with iodine, dissolved in alcoholic caustic soda and estimated as in section 2a.

The percentage of starch found in the barley and malted barley kernals by this method was 66% and 58% respectively. A quantitative yield of starch can be obtained from cereals by this type of chemical treatment of the kernals. The method however cannot be used to

obtain starch in an undegraded granular state and granular starch was isolated from barley and malted barley by the standard methods previously described.

Properties of the granular starches. - Table 4.12 summarises the comparative properties of the two starches. The malted barley starch possessed a higher amylose content, higher gelatinisation temperature and smaller granules than that of the original barley. There was a significant increase in the iodine affinity of the malted barley starch, corresponding to an increase in the amylose content of about 3.5%. This value was in agreement with that reported by previous workers (Aspinall et. al. (1955); Hall et. al. (1956)). As has already been discussed in section 3, the phenomena of gelatinisation is complex. The size of the granules, the molecular size of the components and the degree of crystallinity of the granule may all influence gelatinisation. In this case the smaller granular size and the higher amylose content of the malted-barley granules may cause the increase in their gelatinisation temperature. The distribution curves (Figure 4.3) show that the granules from the malted barley starch are significantly smaller than those of the barley. As both starches were in equilibrium with water when examined, these differences must be significant and are not due to environment.

Fractionation of the starches by aqueous dispersion. - When the components of the two starches were isolated by an aqueous dispersion of the untreated granules, it was found that incomplete dispersion had

FIG. 4·3

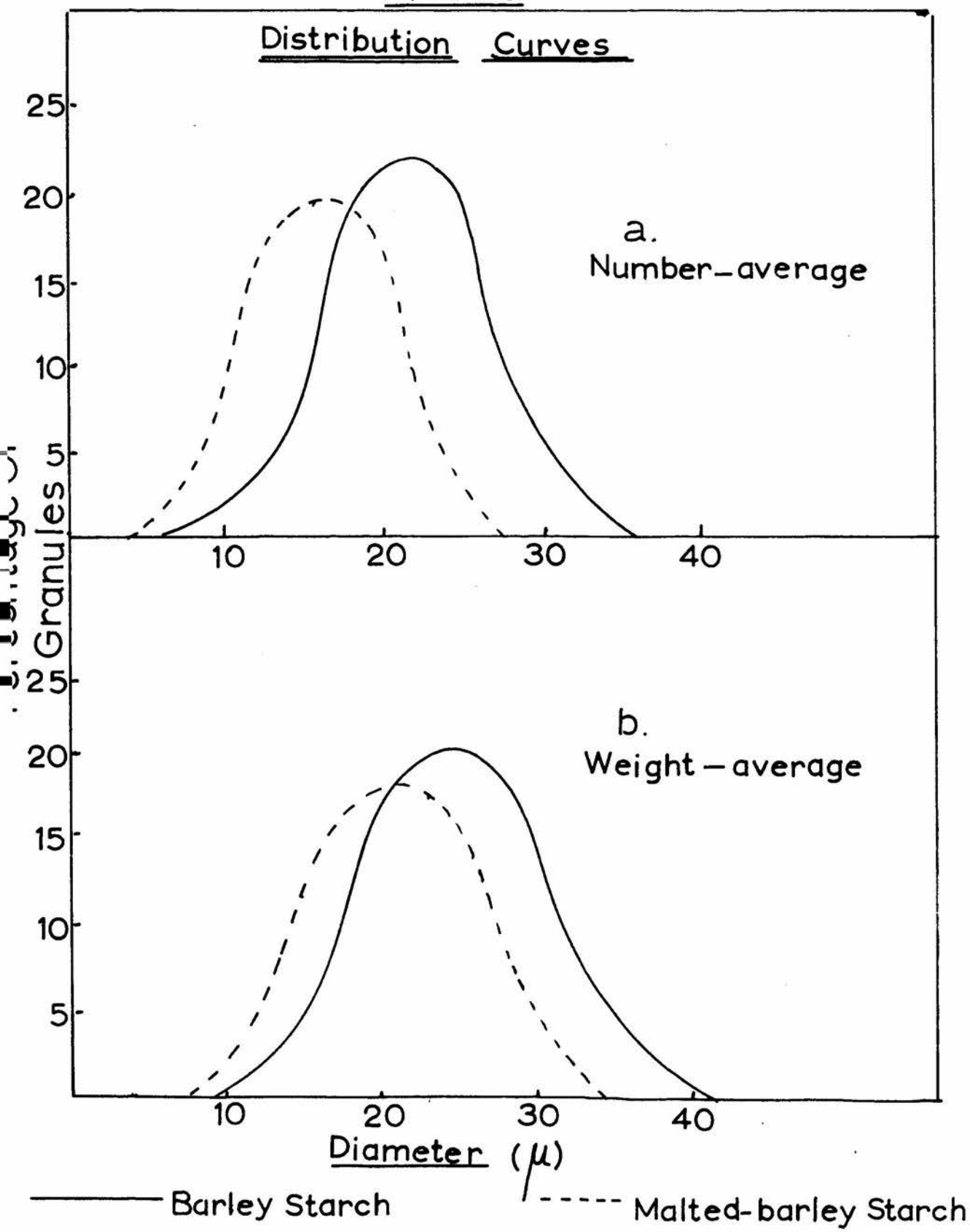

Distribution Curves
25
20
15
10
5
Granules
a.
Number–average
10
20
30
40
25
20
15
10
5
b.
Weight – average
10
20
30
40
Diameter (μ)
Barley Starch
Malted-barley Starch

Table 4.12.

Properties of the granular starches.

Sample	Starch in kernal (%) [a]	I.A. [b]	Amylose [c] %	Gelatinization temp. °C	Size of [d] granules (μ.)
Barley	64	4.25	22.4	59-64.5	(1) 21 (11)24
Malted barley	58	4.95	26.0	64.8-67.5	(1) 17 (11)21

a % dry weight

b IA = Iodine Affinity

c % Amylose = $\frac{\text{I.A. starch}}{\text{I.A. amylose}}$

d Values for (1) Number average (11) Weight average.

had occurred and that the yield of amylopectin from both starches was only one-third of the theoretical. Consequently, the amylose complex was very impure. A similar effect had been observed by Aspinall et. al. (1955). The granules were therefore pretreated with liquid ammonia before dispersion (see section 3). A control experiment on potato and wheat starch was also carried out to ensure that no degradation occurred during this pretreatment. The properties of the fractions obtained, by dispersion of the pre-treated granules are given in Table 4.13.

Properties of the components. - Although the amylopectin from the barley starch was reasonably pure, that from the malted barley (amylopectin 1) was only 75% pure. The addition of more thymol to the amylopectin solution produced no further precipitation of amylose. Butan-1-ol added to the heated amylopectins solution (60°C) caused the precipitation of more amylose-complex. The resultant malted barley amylopectin (2) was 96% pure, while the amylose obtained by this secondary precipitation (Malt amylose 2), had a higher β -amylolysis limit and a lower limiting viscosity number $[\eta]$, than that of the amylose obtained in the original precipitation (malt amylose 1). All the amylose fractions were pure as shown by their iodine affinities and their conversion to maltose under the concurrent action of β -amylase and Z-enzyme. The amylose from the barley starch was larger in molecular size and had a lower β -amylolysis limit than had that from the malted barley. The amylopectins also differed with respect to both

Table 4.13.

Properties of components obtained by aqueous dispersion of starches.

Component	Iodine Affinity	Purity %	Limiting viscosity no. $[\eta]$	Degree of polymerization [a]	β-Amylolysis limit [b]	Unit chain length [c]
Barley amylose	19.0	99	240	1,700	(i) 72 (ii) 98	-
Barley amylopectin	0.38	98	188	-	(i) 58 (ii) -	25.5
Malt amylose 1	19.0	99	200	1,500	(i) 77 (ii) 98	-
Malt amylopectin 1	4.8	75	-	-	(i) - (ii) -	-
Malt amylose 2[x]	19.0	99	115	850	(i) 90 (ii) 101	-
Malt amylopectin 2[x]	0.74	96	146	-	(i) 48 (ii) -	18.4

[a] Degree of polymerisation = $[\eta]$ x 7.4

[b] Expressed as percentage conversion to maltose under the action of (i) β-amylase and (ii) β-amylase + Z-enzyme.

[c] by periodate oxidation

[x] these samples were obtained from amylopectin 1. (see text)

the average length of unit-chain and to the extent of conversion to maltose by β -amylase. The 25-26 unit chain length found for barley amylopectins was similar to that of other amylopectins (Greenwood 1960), but the malted barley amylopectin had the unusual chain length of 18 units (cf. Aspinall et. al. 1955). The β -amylolysis limits of the respective amylopectins also differed. As β -amylase can only degrade the exterior chains of amylopectin and as its action ceases 2-3 glucose units from the α -1:6-inter chain linkage, it is possible to calculate the external and internal chain lengths of the two amylopectins. The external chain lengths of barley and malted barley was found to be 17-18 units and 10.5-11.5 units respectively and the corresponding internal chain lengths 7.5-8.5 and 7-8 glucose units. It was possible that the low chain length observed for the malted barley might be due to a mixture of enzymically degraded and undegraded polysaccharides. In an attempt to show this the amylopectins were subfractionated by preferential precipitation with ethanol. Table 4.14 shows the results of the fractionation which did not result in fractions with significantly different chain-lengths or β -amylolysis limits. A comparison of the molecular size of the amylopectins can be obtained from sedimentation velocity results by ultracentrifugation studies of the two amylopectins in 0.2M-potassium hydroxide solution. The graphs of S_{20}^{-1} against concentration both extrapolated to a sedimentation velocity of 250 x 10^{-13} c.g.s. units thus indicating that the amylopectins were large and comparable in size.

Table 4.14.

Properties of subfractionated amylopectins.

Amylopectin from	Ethanol in mixture (%)	Amylopectin fraction (%)	Unit chain length	β-Amylolysis limit
Barley	20	70	25.8	58
	30	2	25.6	-
	50	3	25.4	-
	50	25	25.5	59
Malted barley	20	74	18.1	48
	30	2	17.7	-
	50	3	17.6	-
	50	21	17.8	48

Successive leaching experiments. - In order to study differences in the amylose from barley and malted barley samples, the starches were leached successively in water at 70°, 80° and 90°C (as in section 3) and the final residue completely dispersed. The characteristics of the components obtained by leaching are given in Table 4.15. As had been previously observed (Cowie and Greenwood 1957), as the leaching temperature increased, the degree of polimerisation of the amyloses increased and their β-amylolysis limit decreased. For both starches leaching at low temperature, yielded material which was completely degraded by β-amylase, while at higher temperatures material of lower β-limit was obtained. As has been postulated by Arbucle and Greenwood (1958), amylose may exist with a β-limit of 50%. Here is some supporting evidence to this hypothesis.

Discussion of results. - From the tabulated results, the general pattern of change in barley starch during malting can be followed. The 16% loss in weight in malting can be accounted for, by the 28% loss in weight observed for the amylopectin component, if this is assumed to be degraded by enzymes to soluble products. Such a degradation would entail a corresponding increase in apparent amylose content to 25.5% in malted barley starch. This agrees with the experimental value of 26%. It seems probable that this degradation to give soluble products is more likely to account for the increase in amylose content than either of the possibilities of synthesis of more amylose or the action of some type of debranching enzyme on the external chains of amylopectin

Table 4.15.

Properties of amylose fractions

(Obtained by successive aqueous leaching of granules followed by dispersion of residue).

Starch	Procedure (temperature °C)	Amylose extracted (% of total)	I.A.	Limiting viscosity no. $[\eta]$	D.P. [a]	β-amylolysis limit [b]
Barley	70° granule leach	26	19.0	90	700	(i) 96 (ii) 101
	80° residue leach	15	18.9	146	1200	(i) 74 (ii) 100
	90° residue leach	20	18.2	246	1800	(i) 65 (ii) 98
	Dispersion of residue	39	17.5	270	2000	(i) 62 (ii) 95
Malted barley	70° granule leach	15	19.0	70	500	(i) 97 (ii) 100
	80° residue leach	16	18.8	130	930	(i) 84 (ii) 100
	90° residue leach	30	18.6	210	1600	(i) 74 (ii) 98
	Dispersion of residue	39	18.0	220	1650	(i) 70 (ii) 98

[a] and [b] As in Table 4.13

resulting in increased linear material. This enzymic degradation would also explain initial loss of starch content, the apparent increase in amylose content and the decrease in the granular size during malting. Microscopic observation of malted barley starch granules, indicated evidence of amylolytic attack similar to that shown in the comprehensive review article by Sandstedt (1954), and it is probable that in the malting process the granules are attacked by both α and β-amylase. The separate components also show evidence of having been subjected to enzymic attack. The observed increase in the β - amylolysis limit for malted barley amylose can be simply explained by postulating α -amylolytic attack on the original barley amylose. For example the molecular size of amylose fraction (2) from malted barley starch (see Table 4.13), corresponds to 1.12 bonds broken per initial amylose molecule with a β-limit of 72%. Assuming the barrier to β -amylolysis is randomly placed in the molecule, it can be calculated that the theoretical limit for 1 bond being broken in such a molecule would be 86%. This is in good agreement with the observed value of 90%. The extent of α -amylolytic activity must however be extremely small. The single chain type of attack of β -amylase on amylose would cause an apparent decrease of the β -amylolysis limit and is therefore unlikely to have occurred. On this basis, it is not necessary to postulate the synthesis in malted barley starch, of amylose with different properties from the original barley amylose. The fact that both the

barley and malted barley amylopectins, have the same internal chain length, suggests that limited β-amylolytic attack must have occurred, while the high molecular weight and comparable size of the amylopectins suggests that no α-amylolytic attack has occurred.

It can therefore be postulated that the enzymic pattern during malting consists simply of (1) limited α-amylolytic attack of the amylose with no β-amylolytic attack and (2) limited β-amylolytic attack of the amylopectin with no α-amylolytic attack.

This type of limited enzymic attack of barley starch during the germination period, when conditions for enzymic attack are perfect, suggests that there must be either an amylase inhibitor liberated during this period, or the granules are undamaged and therefore not accessible to enzymic attack (see Sandstedt 1954).

In order to understand the nature of the enzymic attack a further study was made of the starch components present in the distal and embryo sections of the barley and malted barley grain. The starches were obtained, purified and subjected to conventional dispersion (as in section 3) and the comparative characteristics are shown in Tables 4.16, 4.17, 4.18. The samples used in this study were provided by the Brewing Industry Research Foundation. The variety of malted barley and barley used in this study was Carlsberg. The starches cannot therefore be compared directly with those previously studied as their variety was Ymer.

Table 4.16 shows that the starches, obtained from the distal

Table 4.16

Properties of Granular Starches.

Sample	Portion of grain	Iodine affinity (%) *	Amylose (%) ≠	Gelatinization temperature (0°C)
Barley	Embryo	4.46	23.5	60-62
	Distal	4.16	21.8	61-64
Malted Barley	Embryo	5.10	27.0	64-67
	Distal	4.25	22.1	62-65

* Expressed as mg. I_2 bound/100 mg. starch; titration conditions:- I^- = 0.01M; pH = 5.85; temperature = 20°C.

≠ Calc. from (Iodine affinity of sample)/(Iodine affinity for pure amylose) x 100.

Table 4.17

Properties of Amylose Components Obtained from Aqueous Dispersions of the Starches.

Sample	Portion of grain	Iodine affinity (%)	β-Amylolysis limit *	Purity (%)	Limiting viscosity no. [η]	Degree of polymerization ≠
Barley	Embryo	19.2	(i) 76 (ii) 100	99	220	1600
	Distal	19.1	(i) 71 (ii) 100	99	260	1900
Malted barley	Embryo	19.2	(i) 82 (ii) 101	99	180	1300
	Distal	19.0	(i) 75 (ii) 100	99	240	1800

* Expressed as percentage conversion into maltose under the action of (i) β-amylase, and (ii) β-amylase + Z-enzyme.

≠ Calculated from Degree of polymerization = 7.4 x [η]

Table 4.18.

Properties of Amylopectin Components Obtained from Aqueous Dispersions of the Starches

Sample	Portion of grain	Iodine affinity (%)	Purity (%)	β-amylolysis limit *	Unit chain length /	Internal chain length xx	Limiting viscosity no. [η]
Barley	Embryo	0.42	99	57	24.0	7-8	180
	Distal	0.30	99	60	26.4	8-9	200
Malted barley #	Embryo	0.70	97	48	17.6	6-7	150
	Distal	0.40	98	54	20.0	6-7	210

* Expressed as percentage conversion to maltose under the action of β-amylase.

/ Anhydroglucose units.

xx Calculated from Chain-length − [(chain-length) x (β-amylolysis limit) + 2.5 .]

\# The amylopectins from samples of malted barley had to be [illegible]

and embryo portions of the grain, differed significantly in amylose-content and gelatinisation-temperature. The amylose fractions from barley starch, obtained by dispersion and subsequent fractionation of the embryo and distal starches, were found to differ in β -amylolysis limit and molecular size. These differences are probably related to the maturity of the starch. The properties of the amyloses from the malted barley starches (see Table 4.17) were consistent with a general limited α -amylolytic attack on the barley amylose. The amylopectin fractions from the barley starches differed with regard to chain-length and β -amylolysis limit and as would be expected the properties of the malted barley amylopectins can be accounted for by limited β -amylolysis of the component in the barley.

Although the barley and malted barley used in these experiments was of a different variety (Carlsberg), than those used in the initial comparison (Ymer), it is of interest that the average values for the apparent percentage amylose and the average gelatinisation temperature, for the two halves of the grain are similar to that previously reported. The results in Table 4.16, show that in both the barley and malted barley samples, there is more amylose in the embryo than in the distal section of the grain. Earlier work on starches (Banks and Greenwood 1960), has shown that as the maturity of the starch increased, the β -amylolysis limit of the amylose content decreases. The difference observed in the β -amylolysis limits for the two sections of the ~~two sections of the~~ grain would by analogy to the earlier work, suggest

that the starch in the distal section is more mature.

The work on those differing portions of the grain, while supporting the previous work, also indicates that the starch present in different sections of the cereal grain, are different from each other. Further investigation, which must obviously be made on other starch materials, might profitably yield much information regarding the biosynthesis of the starch granule.

Section 5

The Biosynthesis Of The Starch Granule

5a.

THE PROPERTIES OF THE COMPONENTS OF STARCHES FROM SMOOTH- AND WRINKLED SEEDED PEAS DURING GROWTH

Introduction:-

Mature pea starches have been reported to contain more amylose than the 20-25% usually present in root or cereal starches; there being ca 35% in smooth seeded and ca 70% in wrinkled-seeded varieties (McCready, Guggolz, Silveira and Owens, 1950). Previous work on these starches by Potter, Silveira, McCready and Owens (1953) has indicated that although the amylose components are similar, the amylopectin component from a wrinkled-seeded variety has an abnormal length of unit-chain of 36 glucose units and a smaller molecular size than that from a smooth-seeded pea. These hitherto unconfirmed results suggest that either some radical alteration has occurred in the balance of the synthesising enzymes, or a new type of branch-synthesising enzyme exists.

In the work described in this section the components from mature smooth- and wrinkled-seeded pea starches have been characterized by measurements of extents of conversion by β-amylase, potentiometric iodine adsorption, periodate oxidation, and molecular size. These properties have been compared with those of typical root and cereal starches. Furthermore in an attempt to gain some insight into the method of biosynthesis of the starch granules, the properties of

pea starch and its component amylose and amylopectin during growth have been studied.

EXPERIMENTAL

Isolation of starches. Freshly-gathered peas were exhaustively extracted with 0.01M-$HgCl_2$ and then deproteinized as previously described in section 3a. (It has been found that the starch in dried peas can be similarly extracted and purified if the peas are initially soaked overnight in 0.01M-$HgCl_2$ at room temperature). The purified starches were then exhaustively defatted with boiling 80% aqueous methanol. The varieties of pea investigated and the properties of the isolated starches are given in Table 5.1.

Development of granules. Peas were harvested at various stages of growth and graded in size by sieves before the starch was extracted as above.

Table 5.5 shows the properties of the resultant materials. Percentages of starch were estimated by the method described by MacWilliam, Hall and Harris (1956). Gelatinization temperatures were measured as described previously.

Fractionation of starches. Fractionation was achieved by both conventional dispersion and aqueous leaching experiments (Banks, Greenwood and Thomson, 1959). In agreement with the work of Potter et al (1953), pea starches would not disperse directly into water and pretreatment with liquid ammonia was necessary.

The purity of the separate components were measured by standard potentiometric titration methods and enzymically. Limits of β -amylolysis were measured with purified soya-bean β -amylase and also with mixtures of β -amylase and Z-enzyme. Optical density measurements on polysaccharide - iodine complexes were made with a Unicam spectrophotometer (SP600) under the conditions described by (Hassid and McCready 1943). The average length of unit chain for the amylopectin fraction was determined by periodate oxidation as described in section 2g. Measurement of molecular size. The methods used to measure limiting viscosity numbers $[\eta]$ and sedimentation coefficients S_{20} of the components in alkali have been described in section 2d and 2e. In collaboration with Dr. W. Banks, weight-average molecular weights ($\bar{M}w$) of some amylopectin fractions were obtained from light scattering measurements on the polymers dissolved in water. Measurements were made with a Brice-Phoenix Photometer (Model 1000D) with cylindrical cells and the narrow diaphragm system. Solvent was filtered through sintered glass (G5) directly into the light-scattering cell. Amylopectin solutions (ca. 0.6%) were centrifuged for one hour at 15,000 r.p.m., and successive aliquots of the clarified solution were added to a weighed amount of solvent in the cell to form a concentration series in the range (1-20) x 10^{-5} g./ml. The angular distribution of scattered light was measured over the range 20° - 45° at 546 mu.

Properties of the mature granular starches.

Results and discussions. - Preliminary studies on several varieties of peas (see Table 5.1) confirmed the results of earlier workers, (McCready et al, 1950; Potter et al, 1953), that the smooth-seeded varieties contained more starch (42-43%) than the wrinkled-seeded varieties (31-32%). Although it has been suggested that pea starches are extremely difficult to deproteinized (Senti and Dimler 1959), no difficulty was found using the method described in section 3a. For all starch samples, the nitrogen-content was reduced to a satisfactory low level without treating the granules with any chemical reagents, which might have modified the structure of the starch. Potentiometric iodine titration experiments showed that the amylose content of the starches from the smooth-seeded variety of pea was 34-35%, whilst that of the wrinkled-seeded variety was as high as 65-66%. As shown in Table 5.1, these results have confirmed the earlier observations in the literature. For all the pea starch samples, although the amylose-content varied so much between the two types - the average gelatinisation temperature was greater than 98°. Because of this, the starches would not disperse in boiling water and their behaviour on aqueous leaching was abnormal (see Table 5.7). This gelatinisation behaviour is uncommon. In all the starches studied there has been nothing comparable.

The granules of the starches from the smooth-seeded variety on microscopic examination were found to be "simple" whilst those from wrinkled-

Table 5.1

Varieties of peas and properties of starches

Iodine affinities are expressed as mg. of I_2 bound/100 mg. of starch, % of amylose is calculated from (iodine affinity/19.0) x 100.

Pea		Starch			
Variety	Type	Starch(%)	Nitrogen(%)	Iodine Affinity	Amylose(%)
British Lion	Smooth-seeded	43	0.04	6.6	35
Kelvedon Monarch	" "	42	0.04	6.5	34
Alaska*	" "	-	0.06*	7.1*	34.5*
Gradus	Wrinkled-seeded	32	0.03	12.5	66
Gladstone	" "	31	0.02	12.4	65
Onward	" "	32	0.02	12.4	65
Perfection*	" "	-	0.24*	13.3*	66*

* Results quoted by Potter, Silveira, McCready and Owens (1953).

seeded varieties appeared to be essentially "compound". The morphology of the pea-starch granule is confused however and it is not certain whether true "compound" granules exist (see Badenhuizen 1959). This problem is discussed further below.

Aqueous leaching and dispersion of mature granules. - Starches from two varieties of smooth-seeded pea (British Lion and Kelvedon Monarch), and two varieties of wrinkled-seeded pea (Gradus and Gladstone) were selected for more detailed study. The properties of the starch subfractions obtained by successive aqueous leaching and complete dispersion of the granules were examined.

Leaching of typical cereal and tuber starches at 70-80° has already been shown to result in the preferential extraction of some 20-30% of short-chain, linear amylose. In the case of all the pea starches, however, the amount of extracted amylose was only 2-3% of the total. Indeed, even after leaching successively at 70°, 80° and 90°, more than 90% of the total amylose remained inaccessible. The remarkable contrast in behaviour of the pea starches compared with that for potato and barley starch is shown in Table 5.2. This suggests a profound difference in granular structure which is independent of the percentage of amylose.

As the pea starches would not disperse directly into water, pre-treatment of the granules was necessary. This was successfully accomplished by liquid ammonia as in section 3c. (Potter et al 1953). The properties of the resultant components are shown in Table 5.3

Table 5.2

Properties of amylose fractions obtained by successive aqueous leaching of the granule followed by dispersion.

Procedures I-IV were as follows: granules were leached with water for 10 mins. at 70° (I), 80°(II) and 90° (III). The residue was then treated with liquid ammonia (Banks, Greenwood and Thomson, 1959) and dispersed in water for 1 hr. at 100° (IV). All experiments were carried out in N_2. Amount of amylose extracted is calculated from iodine titration experiments. S = smooth-seeded pea; W = wrinkled-seeded pea.

	Procedure	Amylose extracted (%)	[η]	β-limit*		Procedure	Amylose extracted (%)	[η]	β-limit
British Lion	I	2	110	100	Gradus (W)	I	2	105	100
	II	3	125	88		II	2	115	89
	III	4	170	83		III	3	130	83
	IV	91	190	80		IV	93	150	79
Kelvedon Monarch	I	2	95	100	Gladstone (W)	I	2	95	100
	II	3	120	90		II	3	110	91
	III	3	170	81		III	4	125	83
	IV	92	195	80		IV	91	145	79
Potato (var. Redskin)≠	I	35	250	99	Barley (var. Ymer)≠	I	26	89	96
	II	-	-	-		II	15	146	74
	III	-	-	-		III	20	246	65
	IV	50	570	75		IV	39	272	62

* Expressed as percentage conversion into maltose.

≠ Results from Banks, Greenwood and Thomson (1959).

≠ Results from Greenwood and Thomson (1960).

*** This leaching was at 60°. A further aqueous leach at 63-65° extracted another 15% of the amylose ([η] = 320; β-limit = 82%).

together with the comparative results for potato and barley starch. The iodine affinity and the β-amylolysis limits for the amyloses from both the smooth and wrinkled-seeded pea starches were similar. The incomplete conversion into maltose under the action of β-amylase was not due to contaminating amylopectin, as under the concurrent action of β-amylase and Z-enzyme, conversion was complete. The latter provides a sensitive test for the purity of amylose. The values of the β-amylolysis limits were similar to that found for potato starch but higher than that for barley (see Table 5.3). There was a tendency for the limiting viscosity number for the amyloses from the smooth-seeded pea starches to be lower than that for the wrinkled-seeded samples. This is discussed further below. The values for viscosity are similar to those of Potter et al (1953).

Potentiometric iodine-titrations showed that the amylopectin components were more impure than is usual (see Table 5.3 for potato and barley amylopectins). The titration curves for the British Lion and Kelvedon Monarch amylopectins were normal but the corresponding ones for the Gradus and Gladstone sample, possessed an abnormally large slope for the linear portion of the curve. This made estimation of the amount of contaminating amylose difficult; the value quoted is minimal. As a result, the correction applied for the amylose content is both the β-amylolysis experiments and chain-length determinations are not known with any high accuracy for the latter samples.

Table 5.3.

Properties of components from dispersion experiments on mature smooth and wrinkled pea starches.

Starch	Pea						Potato	Barley
Variety	British Lion	Kelvedon Monarch	Alaska*	Gradus	Gladstone	Perfection*	(Homeguard)	(Ymer)
Type	S	S	S	W	W	W	-	-
Amylose								
Iodine affinity (%)	19.0	19.0	19.0	19.0	18.9	18.7	19.5	19.0
β -amylolysis limit(i)	81	80	-	82	81	-	83	72
β -amylolysis limit(ii)	100	100	-	100	101	-	-	98
[η]	180	170	180	160	130	130	450	240
D.P.	1300	1300	1300	1200	1000	1000	3300	1800
Amylopectin								
Iodine affinity (%)	1.0	1.0	1.6	2.7	2.7	-	0.1	0.38
Purity (%)	95	95	92	86	86	-	99.5	98
β -amylolysis limit (i)	58	57	-	65	66	-	56	58
[η]	150	140	160	120	120	120	160	188
Apparent chain-length	26	27	26.6	36	35	36	24	25.5
Apparent internal-chain length	8	9	-	10	9	-	8	8

* Results from Potter et al (1953)

β -amylolysis limits (i) β -amylase (ii) β -amylase + Z-enzyme.

Results from Greenwood and Thomson (1960)

S = Smooth-seeded pea; W = wrinkled seeded pea.

Calculated from Chain length - [(chain length x β -limit) + 2.5]

The values of β -amylolysis (57-58%) and chain-length (26-27 anhydroglucose units) for the amylopectins from the smooth-seeded peas are comparable to those reported earlier for amylopectins. The corresponding values of 65-66% conversion into maltose and an apparent chain length of 35-36 anhydroglucose units for the wrinkled seeded samples are abnormal, but agree with the earlier unconfirmed report by Potter et al (1953). Calculations showed that within experimental error, the internal chain-lengths of all samples were the same. This suggests, in conjunction with the extra apparent amylose, the presence of a new or modified branching enzyme in wrinkled-seeded varieties of pea. These samples were therefore examined in detail.

Properties of amylopectins from mature pea starches. - When the abnormal amylopectins were studied in alkaline solution in the ultracentrifuge, however, they were found to be inhomogeneous. Both the Gradus and Gladstone amylopectins consisted of a rapidly moving component (S_{20} ca several Hundred), together with a large amount of material with a very low sedimentation coefficient (S_{20} 1). The British Lion and Kelvedon Monarch amylopectins behaved normally. Differential ultracentrifugation carried out on the abnormal samples, enabled the two components to be separated. Table 5.4 shows the results, together with the control experiment on British Lion amylopectin. It can be seen that the sedimented components from both "36-unit" amylopectins have a chain-length and β -amylolysis

Table 5.4.

Some properties of the amylopectins and subfractions of the amylopectins from pea starches.

0.2 aqueous solutions of the amylopectins (obtained from a dispersion) were spun for two periods of 2 hr. at 44,000 r.p.m. in the preparative rotor of the Spinco Model E ultracentrifuge. Material was isolated from the supernatant liquor by freeze-drying. Apparent purity of the amylopectin-fraction was obtained by iodine titration. W = wrinkle-seeded pea; S = smooth-seeded pea.

Pea	Type	Component	Amount (%)	Apparent purity (%)	Apparent chain length	β-Limit	[η]
Gradus	W	Original	100	86	36	65	120
		Sediment	80	91	27	57	150
		Supernatant	20	-	-	100	35
Gladstone	W	Original	100	86	35	66	120
		Sediment	-	91	28	58	140
		Supernatant	-	-	-	100	
British Lion	S	Original	100	95	26	58	150
		Sediment	-	97	27	57	160
		Supernatant	-	-	-	-	-

limit comparable to that for the original British Lion amylopectin when corrections were made for apparent purity. The properties of the latter were effectively unaltered on differential ultracentrifugation, although some amylose was removed. The materials isolated from the supernatant liquors of the Gradus and Gladstone samples were each completely degraded into maltose by β-amylase and had very low limited viscosity numbers. These materials had apparently the properties of degraded, short-chain, linear amyloses.

These results suggest, that the abnormal amylopectins are thus artefacts caused by the presence of contaminating, soluble, short-chain amylose in an otherwise normal amylopectin. It is not necessary to suggest that a radical upset has occurred in the starch-synthesising enzymes, nor is another new enzyme necessary. Further aspects of the implications of these results are discussed below.

Molecular weight of the components from mature starches. - Molecular weights for two of the amylose samples were obtained from sedimentation and viscosity measurements using the equation of Scheraga and Mandelkern (1953) i.e.

$$M = \left[\frac{N\,\eta_o}{\beta(1-\bar{V}\rho)}\right]^{3/2} S_o^{3/2}\,[\eta]^{1/2}$$

Here, η_o = the solvent viscosity; N = Avogadro's Number; $\bar{V}$ = the partial specific volume of solute; ρ = the solvent density; S_o = the sedimentation coefficient at infinite dilution;

$[\eta]$ = the limiting viscosity number; β = a constant related to the molecular shape. Sedimentation and viscosity measurements were made on aqueous solutions of the amyloses from British Lion and Gradus pea starches. The results were as shown.

British Lion :- $[\eta] = 180$; $S_{20} = 7.1 \times 10^{-13}$

Gradus :- $[\eta] = 140$; $S_{20} = 6.6 \times 10^{-13}$

Hence using a value of $\beta = 2.25 \times 10^{6}$ and $\bar{V} = 0.65$ (Cowie 1958.) the molecular weights were

British Lion amylose:- $M = 1.15 \times 10^{6}$; D.P. = 7000

Gradus amylose :- $M = 1.05 \times 10^{6}$; D.P. = 6400

These values for the degree of polymerization are much larger than earlier values in the literature (see Table 5.3) because the latter were number-average values and the calculated ones are more nearly weight-average values. The determination of the molecular weight of amylopectin is difficult. The earlier values reported in the literature (Potter et al 1953) are not likely to be very accurate as osmometry experiments on such high molecular weight materials are difficult. Furthermore the probable contaminating short-chain amylose in the original sample of amylopectin from wrinkled-seeded peas would make molecular weight determinations inaccurate. In this work, viscosity measurements (Table 5.3) indicated that the amylopectins from all varieties were comparable. This conclusion was confirmed by lightscattering measurements made by Dr. W. Banks. Molecular weights

of 1.5×10^8 and 1.4×10^8 for the Gradus and British Lion amylopectins respectively, were obtained.

Growth experiments. - Table 5.5 shows the properties of the granular starches isolated at various stages of growth of smooth-seeded and wrinkled-seeded varieties of pea. With increase in maturity of the pea, there is an increase in the percentage of starch in both varieties. The properties of the isolated starches show that with increase in maturity there is an increase in (i) granule size (ii) average gelatinisation temperature and (iii) the percentage of amylose. On microscopic examination there were obvious differences between the granules at different stages of growth. The granules in the smooth-seeded British Lion starches were oval but somewhat irregular structures, the average size of which increased with maturity. The granules of the wrinkled-seeded Gradus starches were smaller and round. In the earlier stages of growth there was no evidence of compound granules; these appeared during the final stages. In the mature Gradus starch, as well as the compound granules there were a large number of small spherical granules. Some granules showed evidence of enzymic attack and the larger granules were deeply fissured. The problem of the nature of pea starch granules is not simple (see Badenhuizen, 1959).

Properties of starch components during growth. - The starch samples isolated during growth were fractionated. The properties of the separated components are shown in Table 5.6. The complete conversion

Table 5.5

Properties of the granular starches obtained during growth.

	Pea			Starch			
	Diameter (mm)	Starch[a] (%)	Sample	Average granule size[b]	Granule type[c]	Average Gelatinisation temp(°C)	Amylose[d] (%)
British Lion	2-6	22	S1	10	S	62	15
	6-9	26	S2	15	S	81	19
	9-11	32	S3	30	S	92	29
	11-12	40	S4	40	S	98	37
Gradus	2-6	12	W1	7.5	S	64	23
	6-9	16	W2	12	S	83	36
	9-11	22	W3	20	S/C	97	59
	11-12	31	W4	30	C	98	69

a By method of MacWilliam et al (1956).

b Estimated from photomicrographs.

c S = Simple C = Compound

d $\%\ \text{Amylose} = \frac{\text{Iodine Affinity of starch}}{\text{Iodine Affinity of amylose}}$

Table 5.6

Variation in properties of starch components during growth of peas.

Sample	S1	S2	S3	S4	W1	W2	W3	W4
Amylose								
β -limit (i) [a]	90	86	79	74	90	82	80	74
β -limit (ii) [a]	100	101	100	100	101	100	100	101
	145	160	195	210	120	130	135	150
Amylopectin								
Purity (%)	98	96	95	92	90	89	86	83
Apparent chain-length	24	26	26	27	29	31	33	37
β -limit (i)	54	56	56	58	58	60	64	66
Apparent internal chain-length	9	9	9	9	10	10	10	10

a Percentage conversion into maltose by (i) β -amylase (ii) β -amylase + Z-enzyme.

b See Table 5.3.

Table 5.7

Amylose

(Obtained by successive aqueous leaching of granules followed by dispersion of residue)

Starch	Procedure (Temp. °C)	Amylose extracted (% of total)	I.A.	Limiting Viscosity no. [η]	D.P.[a]	β-amylolysis limits [b] (1)	(2)
Smooth Pea	70° granule leach	2.0	19.0	100	800	100	101
	80° residue leach	3.0	19.0	130	900	88	100
	90° residue leach	4.0	19.1	170	1300	83	100
	Dispersion of residue	91.0	19.0	190	1400	80	100
Wrinkled Pea	70° granule leach	1.5	19.0	110	800	100	101
	80° residue leach	2.0	19.0	115	850	89	100
	90° residue leach	3.0	19.0	130	1000	83	100
	Dispersion of residue	96.0	18.9	150	1100	79	100

a D.P. = $7.4 \times [\eta]$

b Conversion into maltose by (i) β-amylase and (ii) β-amylase + Z-enzyme.

of the amylose fractions into maltose under the concurrent action of β -amylase and Z-enzyme, shows that they are not contaminated with amylopectin. Consequently the results show that as the pea starches mature, the β -amylolysis limit of the amylose decreases i.e. there is an increase in the extent of the natural barrier to β -amylase in amylose, with maturity. The apparent β -amylolysis limit for any sample of amylose depends therefore on the maturity of the botanical source of the starch. Also during growth, the size of the amylose increases as shown by the increase in limiting viscosity number. The apparent molecular size of any sample of amylose is dependent, therefore on the sample maturity and it is unlikely that amylose samples from different varieties of the same botanical source, will be found to have the same molecular weight.

The amylopectin components isolated from the smooth-seeded peas show small differences in chain length and β -amylolysis limit with increase in maturity (see Table 5.6). The samples from the wrinkled-seeded pea show a gradual increase in both β -amylolysis limit and chain-length. Calculation shows that the apparent internal chain-lengths remain constant, and the implication, might be drawn, that an enzyme capable of forming longer branches on normal amylopectin, might be acting. However the experiments described above show that the presence of short-chain amylose accounts for these results. It is therefore likely that the amount of this short-chain amylose increases with maturity. How far this is related to the

apparent microscopic appearance of enzymic attack on the granules is not known. The presence of degraded amylose may be due to limited α-amylolytic attack, or a partial inhibitor for amylose synthesis may be present. In the first case, preferential α-amylolysis of amylose might well occur as it does during the malting of barley.

Biosynthesis Of The Starch Granule.

Little is yet known of the Biosynthesis of starch granules. Electron microscope studies (Whistler and Thornberg 1957), have shown that granules originate in the amyloplasts. Wolf et al (1948) studied the general properties of maize starch granules during growth and observed an increase in average granule size (see section 1), and an apparent increase in amylose content with maturity. Harris and MacWilliam (1958) have shown that during the growth of barley the starch content increased from 2 - 66% whilst the apparent amylose content of the starch increased from 14 - 26%. A similar increase in amylose content during the growth of maize has been reported by Erlander (1960). Tracer techniques have been used to study the formation of wheat starch (McConnell et al 1958) and potato starch (Badenhuizen and Dutton 1956). In agreement with these earlier results, our studies on pea starches, show that an increase in granular size, gelatinization temperature and percentage of amylose component occurs with increase in maturity.

There are few studies on the actual changes in fine-structure of the components during growth. Such studies are important as they may show changes in enzymic activity. Furthermore there is no evidence as yet that the physical properties of starch products synthesised in vitro are exactly comparable to those of the natural products. Banks and Greenwood (1959) suggested that in potato starch, maturity may result in an increase in molecular size and a decrease in

the β -amylolysis limits for the amylose components was found. These results suggest that either amylopectin is laid down first and the amylose component is subsequently formed, or the amylose is formed from the amylopectin. It is not easy to distinguish between these alternatives.

Section 1 has dealt with the theories proposed for the enzymic synthesis of starch but it is doubtful if a satisfactory theory of enzymic synthesis, which can explain the observed data, has yet been proposed. As has been seen Whelan (1958) has proposed a scheme in which amylose is synthesised by phosphorylase, and amylopectin synthesised by Q-enzyme and D-enzyme together, the two systems being separated by a semi-permeable membrane but drawing on a common reservoir of glucose - 1 - phosphate and maltodextrin primers. The above data for pea starches does not suggest the preferential synthesis of amylose, and in Whelan's scheme it is difficult to see why the percentage of amylose should vary with maturity. Erlander (1958) proposed another scheme in which starch is synthesised via glycogen i.e. plant glycogen is attacked by a theoretical debranching enzyme which (a) removes the outer or available branches of the glycogen to form amylopectin and then (b) connects these removed branches end-to-end to form amylose. Some results of growth experiments can be explained on this basis (Erlander 1960).

The starch from smooth-seeded and wrinkled-seeded peas also forms useful material for genetic experiments. Kellenbarger et al

(1951) have already reported cross-breeding experiments which show that both starch-content and amylose-content obey Mendelian Laws, and that the differences are probably due to a single gene pair. However the above results suggest that the starch in wrinkled-seeded pea is probably subject to abnormal α-amylolytic activity and the factors controlling this are not yet known.

5b.

The Effect Of Growth On The Properties Of Potato Starch

Introduction. - Relatively few chemical investigations have been carried out on the variations in characteristics of the starch components during growth (see Greenwood 1956). Meyer and Heinrich (1942) studied variations in amylose content of starch present in the leaves, shoots and tubers of potato. The percentage of amylose determined by potentiometric iodine titration of the starch in growing tubers was shown by Halsall et al (1948) to remain constant at 17% for two varieties. There is however evidence that the percentage of amylose may vary with the botanical variety of the tuber. Anderson and Greenwood (1955) found small variations in the iodine affinity of 3.94 - 4.03%, representing changes of 20-21% of amylose, and Doremus, Crenshaw and Thurber (1951), reported variations of 17.5-21.7% in 22 varieties of sweet potato. Banks and Greenwood (1959) studied the physical and chemical characteristics of the starch isolated from the shoots and tubers of the sprouting potato and compared them with the original tuber starch. To date no detailed work has been published on the starch present in the tubers at various stages of growth. Erlander (1960) has studied the production of amylose and amylopectin in potato tubers, but has made no effort to relate any changes noted, to the size of the potato tubers or the size of the starch granules. In the work reported here, the physical and chemical characteristics

of the starch isolated from different sizes of potato tubers are reported and considered in the light of current theories of starch biosynthesis.

Experimental

Isolation of the starches. - Potatoes (var. Duke of York) were grown by the Scottish Plant Breeding Station. They were harvested at various intervals and the tubers separated into sizes, varying from potatoes smaller than 1 cm. to potatoes 8-9 cms. in diameter. The starches were isolated as in section 3a and characterised. The sizes of the potatoes investigated and the properties of the isolated starch are given in Table 5.8. The starch granules were characterised by, size distribution, potentiometric titration and gelatinisation temperature.

Fractionation of the starches. - Each starch was fractionated by conventional dispersion (see section 3c). The starches were defatted with boiling methanol and pretreated with liquid ammonia, prior to dispersion.

Characterisation of the fractionated products. - The purity of the amylose and amylopectin components was obtained by potentiometric iodine titration (see section 2b). Limiting viscosity numbers were carried out in 1$\bar{M}$ KOH at 22.5°C as described in section 2d. The percentage conversion of both amylose and amylopectin into maltose was determined as described previously (section 2c); the

enzyme was free from maltase and α -amylase activity and contained no Z-enzyme. Average lengths of unit chain for the amylopectins were calculated from the results of potentiometric estimation of the formic acid released on oxidation with $NaIO_4$ at 2° (section 2g).

If changes in characteristics are to be related to possible biosynthesis methods, the amount of phosphorus in the separate starch components has to be accurately determined. This was estimated as in section 2h.

Results And Discussion.

Physical characteristics of the starches. - The general characteristics of the starches are given in Table 5.8. As the potato increased in size there was a general increase in granular size and amylose content. Unlike pea starch however, the gelatinisation temperature remained reasonably constant, and was not effected to any extent by the variation in amylose content.

Conventional fractionation. - The physical characteristics of the starch components are given in Table 5.9. As can be observed, the amylose fractions were all obtained free from any contaminating amylopectin. However the amylopectins varied considerably in their state of purity. The amylopectin from the smallest potato contained about 12% of amylose. As the size of granules increased, the amylopectin purity increased (to 99%), although the biggest potato size again gave an impure amylopectin with about 4% amylose impurity. The large impurity, found in the amylopectins from the small potatoes is probably due to the fact that the amylose at this stage of biosynthesis is small and therefore not completely precipitated with thymol after conventional dispersion.

Characteristics of the amylose component. - Each amylose was free from amylopectin, as shown by potentiometric titration and enzymic degradation. Viscosity measurements show that the limiting viscosity number $[\eta]$ increases with maturity from 100 in the smallest potato to 450 in the largest potato. Those viscosities correspond to a

Table 5.8

The properties of the starches isolated from various sizes of potatoes.

Size of potato (cms)	Size of granules[a] (μ)	Iodine Affinity[b]	Gelatinisation temp (°C)
→ 1	18	2.95	52-57
1 - 2	22	3.35	-
2 - 3	29	3.55	-
3 - 4	34	3.75	-
4 - 5	38	4.30	54-57
6 - 7	46	4.36	-
7 - 8	-	-	-
8 - 9	54	4.42	54-56

a Average size of granule, determined from size distribution curves obtained by measurement from enlarged photomicrographs of at least 500 granules.

b Iodine affinity measured by potentiometric titration as in section 2b.

Table 5.9

The properties of the components of the starches isolated from various sizes of potatoes.

Size of potato	1	1 - 2	2 - 3	3 - 4	4 - 5	6 - 7	7 - 8	8 - 9
Amylose								
Iodine affinity	19.0	19.0	19.1	19.1	19.2	19.0	19.0	19.1
β -amylolysis limit (i)[a]	92	86	84	84	83	79	-	72
β -amylolysis limit (ii)	100	101	100	100	100	100		100
$[\eta]$	100	130	160	240	290	360	430	450
D.P.	750	1000	1200	1800	2200	2700	3200	3400
% Phosphorus	0.00	nil	nil	nil	nil	0.002	0.004	0.005
Amylopectin								
Iodine affinity	2.10	0.45	0.25	0.20	0.20	0.20	0.28	0.75
β -amylolysis limit (i)[+]	49	52	-	-	56	57.5	-	58.6
Chain length	21.0	22.4	-	-	24.6	25.0	-	26.0
Internal chain length[b]	8-9	8-9	-	-	8-9	8-9	-	8-9
% Phosphorus	0.029	0.039	0.042	0.045	0.048	0.049	0.049	0.049

a Percentage conversion into maltose by (i) β -amylase
(ii) β -amylase + Z-enzyme

b Calculated from Chain length - $\left[\text{(Chain length} \times \beta\text{-limit)} \div 2.5 \right]$

\+ Corrected for amylose present.

change in the degree of polymerisation from 750 to 3400 anhydro-glucose units. This increase in the amylose size was accompanied by a decrease in β -amylolysis limit from 92% in the smallest amylose to 72% in the largest. It is interesting to note that no organic phosphate was found in any of the amylose fractions up to a 4 - 5 cm. potato, but phosphate was found in increasing quantity in the 6 - 9 cm. potatoes. The unknown barrier to β -amylolysis is not therefore caused by the presence of organic phosphate, although this may constitute a barrier in the larger amyloses. Indeed the barrier present in amylose may be caused by branching of the molecule and by the presence of organic phosphate (Greenwood 1960).

Characteristics of amylopectin. - Table 5.9 shows changes occurring in the amylopectin component with growth. Enzymic limits, using purified soya-bean β -amylase show that as the potato increases in size the β -amylolysis limit also increases from 49-58.6%. At the same time the average chain length of the amylopectin increases from 21-26 units. It is interesting to note that this increase in chain length is caused by an increase to the external chain length, as the internal chain length remains constant at 8-9 units.

Estimation of the organic phosphate content showed that the phosphate content increased in the amylopectin as the potato increased in size, up to a 6-7 cm. potato, but in the 7-9 cm. potatoes the phosphate content was almost constant. This factor is of interest when the various theories of biosynthesis of the granule are being compared.

Biosynthesis of Starch.

As the starch granule increases in size, the amylose increases in size and the amylopectin components increase in chain length. In the amylose molecule this increase in molecular size occurs concurrently with an increase in the apparent barriers present in the molecule, to β-amylase action. As these barriers are obviously growth effects, the theory held by several workers that they are artefacts introduced by dispersion methods is probably erroneous. However the changes occurring in growth do not make it possible to distinguish between Whelan's (1958) and Erlander's (1958) hypothesis of biosynthesis. The increase in amylose size is common to both hypotheses. Changes in the chain length of the amylopectin molecule are however not common to both hypotheses. By Whelan's theory it is obviously possible to visualise an increase in the chain length of the amylopectin with growth. Erlander's theory requires the enzymic debranching of a glycogen-type of molecule, and it would therefore be expected that the total chain length would remain reasonably constant. Changes in the β-amylolysis limit of the amylopectins can be explained by both theories. If Erlander's theory is correct, a certain amount of information with regard to the nature of the hypothetical debranching enzyme can be obtained from the phosphate estimations. The approximately constant phosphate-content of the amylopectins in the 6 to 9 cm. potatoes and the introduction of phosphate in the amyloses of these potatoes, can be explained by a steric hindrance of the debranching enzyme, which causes the enzyme to remove internal branches containing pre-formed phosphate groups.

Summary

Current concepts in the chemistry of the starch-type polysaccharides with particular reference to their fine structure, have been critically discussed. The enzymic and physical techniques used in this work are detailed and an enzymic assay for the determination of the purity of amylose has been demonstrated.

A critical examination of the factors influencing the purity of the isolated starches, and the methods of fractionating starch has been undertaken. The necessity for an inert atmosphere in starch fractionating procedures has been illustrated, and the fractionation of potato starch by centrifugation in alkali has been completed and shown to be inefficient. The success of the method of fractionating amylose from dimethyl sulphoxide has been demonstrated.

A number of starches from a wide variety of botanical sources have been isolated and characterised. Pretreatment of the granules with liquid ammonia was found to be a general method of ensuring satisfactory fractionation. A comparison of barley and malted barley of the same variety, showed that during the malting process, the amylose and amylopectin components of the barley starch are preferentially attacked by the amylase enzymes. Floridean starch has been isolated, purified by differential ultracentrifugation and shown to have an amylopectin-type structure.

Finally a critical comparison of the components from smooth and wrinkled pea starch has been completed. The changes occurring in the

structure of the components of pea and potato starch during growth, have been related to changes in granular size and considered in the light of several hypotheses of starch biosynthesis .

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